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13. ABSTRACT (Maximum 200 Words)

We demonstrated that ß-lapachone (ß-lap) was an effective agent against human prostate cancer (CaP) cells/tumors alone. ß-Lap kills by targeting elevated NQ01 levels in CaP tissue. ß-Lap radiosensitizes cells expressing this two-electron reductase; inducible NQO1 levels were sufficient, but not required. Normal cells with low or no NQ01 levels were spared from ß-lap cytotoxicity.

Mechanistically, ß-lap-induced cell responses and cell death with without IR, using cultured human CaP cells *in vitro* were completed. ß-Lap alone kills by stimulating calcium-dependent PARP-1 hyperactivation, resulting in AIF-mediated, caspase-independent cell death. In Aim #2, the efficacy of radiosensitization by ß-lap *in vivo* was examined using human CaP xenografts in male nude mice. To date, we have been unsuccessful in developing LNCaP xenografts from cells expressing or lacking NQO1 in athymic male mice. We are developing stable DU145 and PC3 cells expressing siRNA to NQOO1 to knockdown NQO1 levels. Preclinical animal studies to move ß-lap from the bench to clinical trials against CaP are underway. ß-Hydroxypropyl-ß-cyclodextrin-ß-lap (HP-ß-CD-ß-lap) complexes were developed. However, unexpected neural toxicity at high doses of ß-lap were noted. ***Finally, we developed novel millirods made of PLGA-PEG polymers for use during brachytherapy, and future studies will focus on these localized treatment vehicles.***

14. SUBJECT TERMS

Prostate Cancer, Beta-Lapachone, Calpain, NQ01/X1P8, apoptosis-inducing factor, Millirods, beta-cyclodextrin, Microspheres, millirods, brachytherapy

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David A. Boothman

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PI - Signature **Date:** April 23, 2004

Table of Contents

INTRODUCTION: *Narrative that briefly (one paragraph) describes the subject, purpose, and scope of the research.*

ß-Lapachone (ß-lap), an ortho-naphthoquinone found in the bark of the South American Lapacho rainforest tree, appears to be a promising agent for the treatment of prostate cancer (CaP) cells. CaP is a disease of slow growing neoplastic cells. Agents that kill CaP cells independent of cell cycle status or hormonal regulation are needed. ß-Lapachone is a novel apoptotic inducing agent that: **(a)** kills CaP cells independent of androgen-response, p53 or pRb status, and cell cycle phase; quiescent CaP cells are killed as effectively as cycling cells; **(b)** causes lethality only in CaP cells expressing NADP(H):quinone oxidoreductase (NQ01), an ionizing radiation (IR)-inducible two-electron reductase that is also elevated in many human CaP cells; and **(c)** causes an unique form of apoptosis in CaP cells that is caspase-independent and mediated by activation of calpain or a calpain-like protease. Since CaP may be a disease of abnormal (i.e., blocked) apoptosis, rather than altered proliferation (Wertz and Dixit, J. Biol. Chem. 275: 11470-11477, 2000), ß-lapachone could be an agent ideal for this disease.

ß-Lapachone was originally discovered for its ability to radiosensitize a variety of human cancer cells. Without knowledge of its mechanism of action, however, the compound remained unexplored for use against CaP. Recent elucidation of the mechanism of action of ß-lapachone, showing that this compound is specifically activated by X-ray-inducible NQ01, now allows us to explore the preclinical potential of ß–lapachone (or more efficient analogs), with or without IR. **We hypothesize that ß-lapachone is an effective agent against CaP cells due to its ability to kill cells by a target that is elevated in CaP tissue. Furthermore, the compound will be even more effective in CaP tissues that up-regulate its activating enzyme, the IR-inducible NQ01/xip3 oxidoreductase enzyme. Normal cells with little or no NQ01 levels will be spared, while CaP cells expressing NQ01 (or which induce NQ01) will be killed by a novel form of apoptosis induced by ß-lapachone futile cycling. CaP cell killing will be independent of p53, pRb, cell cycle status, or hormone (non)responsiveness.** We are testing this theory using LNCaP cells that are deficient in NQ01, and we are comparing their responses to cells constitutively expressing NQ01 (i.e., DU145, PC-3 and NQ01-transfected, LNCaP cells) in the presence or absence of inhibitors of NQ01 (e.g., dicoumarol). We examined whether constitutive or inducible NQ01 levels are required for radiosensitization by ß-lap using conditional (doxycycline- or ecdysone-inducible) NQ01 expressing stable cell lines of LNCaP. In Specific Aim 1, we examined *in vitro* responses and mechanisms of cell death after ß-lap, with or without IR, using human CaP cells in culture. In Specific Aim #2, we examined the efficacy of radiosensitization by ß-lap *in vivo* using human CaP xenografts in male nude mice. Our studies provide needed preclinical mechanistic data to move ß-lap, or one of its analogs, from the bench to clinical trials against CaP in the next three years. Such phase I studies with ß-lapachone can be performed with or without IR.

BODY OF GRANT UPDATE: *This section shall describe the research accomplishment associated with each Task outlined in the **approved** Statement of Work. Data presentation shall be comprehensive in providing a complete record of the research findings for the period of the annual/final report. However, appended publications and/or presentations **MAY** be substituted for a detailed description but **MUST** be referenced in the **BODY** of the report. If applicable, for each Task outlined in the Statement of Work, reference appended publications and/or presentations for details of result findings and tables and/or figures. The report shall include negative as well as positive findings, and also shall include any problems in accomplishing any of the tasks. Statistical tests of significance shall be applied to all data whenever possible. Figures and graphs referenced in the text shall be appended. The discussion shall include the relevance to the original hypothesis. Recommended changes or future work to better address the research topic may also be included, although changes to the original statement of work must be approved by the Grants Officer.*

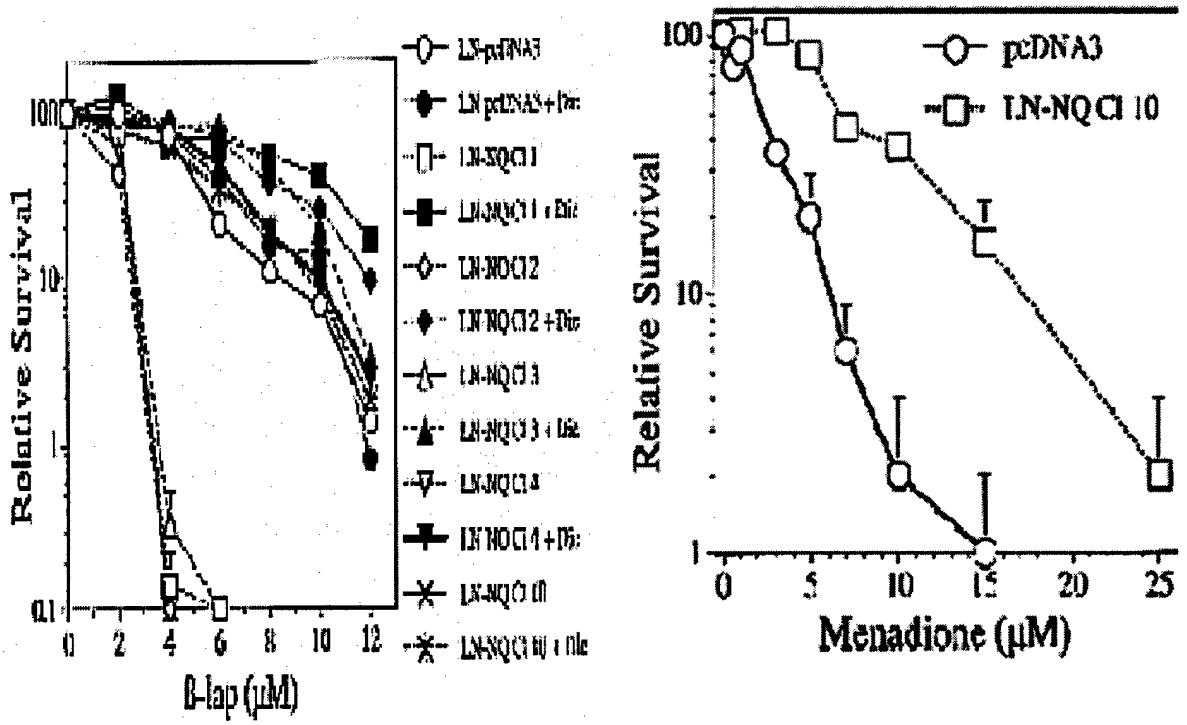
F:* Previous *Statement of Work and Accomplishments Made.

TASK 1: Investigation of whether inducible or constitutive NQ01 levels are required for the ability of ß-lapachone to radiosensitize human prostate cancer (CaP) cells **(Years 0-3). Completed.**

-Develop constitutive NQ01-expressing LNCaP cells in culture.

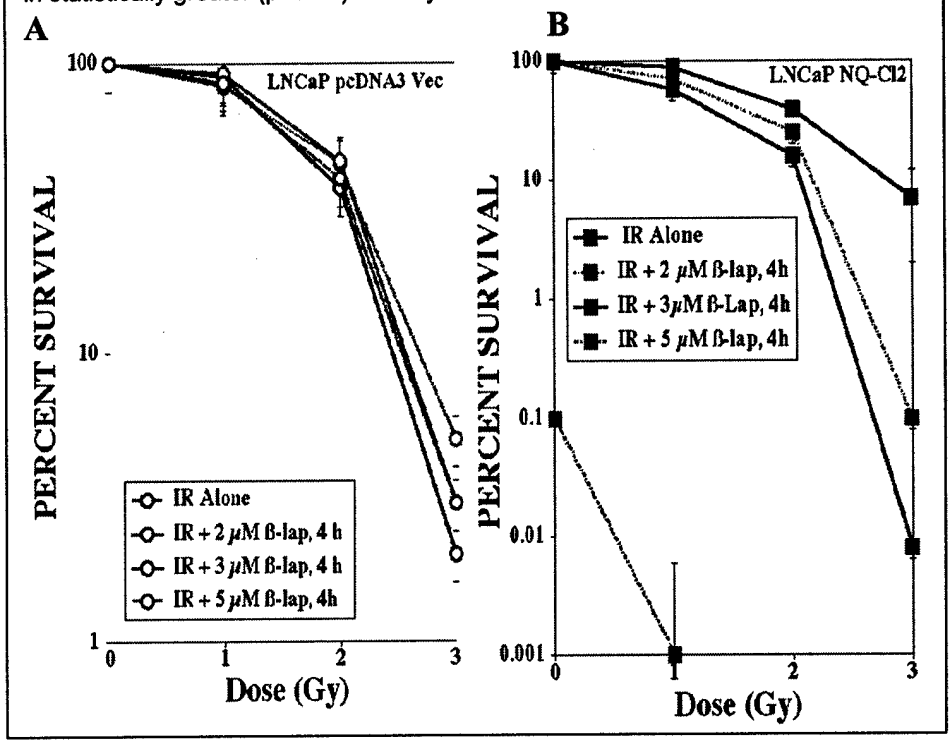
Progress: We established and published LNCaP cells that constitutively express NQ01 and demonstrated that they are responsive to ß-lapachone, whereas, vector alone expressing LNCaP cells are not responsive to the drug. These results were published in our recent paper in Experimental Cell Research (Planchon et al., Exp. Cell Res., 2001): Figure 1 shows how all of the NQ01-expressing cells were killed by ß-lapachone, whereas, vector-alone cells were spared. Furthermore, we demonstrate that NQ01-expressing cells are spared from ß-lapachone cytotoxic responses by co-administering dicoumarol, a specific inhibitor of NQ01.

Figure. 1. **NQO1 enhances ß-lapachone, but decreases menadione, cytotoxicity.** **Left,** NQO1-containing (LN-NQ Cl1-4, 10) and -deficient (LN-pcDNA3) LNCaP clones were treated with 4-h pulses of various doses of ß-lapachone, ± 50 µM dicoumarol. Colony forming ability assays (CFAs) were performed three times, each in triplicate. Open symbols: ß-lapachone alone; Closed symbols: ß-lapachone + 50 µM dicoumarol. **Right,** one NQ01-transfected LNCaP clone (LN-NQ Cl 10) and the LNCaP vector alone clone (LN-pcDNA3) were treated with 4-h pulses of various doses of menadione and CFA assays were determined [45,60].



-In Aim 1, we indirectly tested the hypothesis that NQO1 must be IR-inducible by examining LNCaP cells that constitutively express NQO1 compared to cells that lack expression of this two-electron reductase. We wanted to know if ß-lapachone required NQO1 expression for radiosensitization. LNCaP cells lacking NQO1 (vector alone) and several NQO1-expressing LNCaP clones were exposed to various doses of IR (i.e., 0, 1, 2, 3, and 5 Gy) and then treated with 0.01% DMSO (vehicle) in PBS alone or with ß-lapachone in 0.01% DMSO for 2h. In a series of separate studies we demonstrated that a 2h treatment of ß-lap was sufficient to cause the most dramatic apoptotic responses in NQO1 cells with minimal responses (no lethality) in NQO1-deficient cells. To answer the question of whether NQO1 levels must be induced for ß-lap radiosensitization, we examined $NQO1^+$ LNCaP NQCl2 v. $NQO1^-$ LNCaP-pcDNA3 vector alone cells for differences in radiosensitization by various concentrations of ß-lap, given 4 h post-IR (Fig. 2). Several NQO1+ LNCaP cells were tested and all demonstrated similar responses as noted in Fig. 2. Dose-response clonogenic survival assays revealed two basic conclusions. First, only $NQO1^+$ LNCaP cells were radiosensitized by ß-lap treatments (Fig. 2B). Second, expression of NQO1 alone did not confer radioprotection of cells; it was possible that IR-induction of NQO1 (a.k.a., xip3) would confer a survival advantage of cells following IR exposures. Finally, if the dose of ß-lap was too high (i.e., 5 μM, Fig. 2B), radiosensitization was abolished due to the cytotoxicity of ß-lap alone. Similar results were found in isogenic NQO1+ v. NQO1- breast and nonsmall cell lung cancer cell lines, suggesting that the radiosensitization of cells that express NQO1 was a universal response rather than a unique response noted in CaP cells. **Thus, NQO1 expression was necessary for radiosensitization, and induction of the enzyme is not required for enhanced cell killing of IR-exposed cells by ß-lap.** This is important since NQO1-overexpressing CaP tumors should be sensitized by ß-lap whether they induce, or already express, this enzyme.

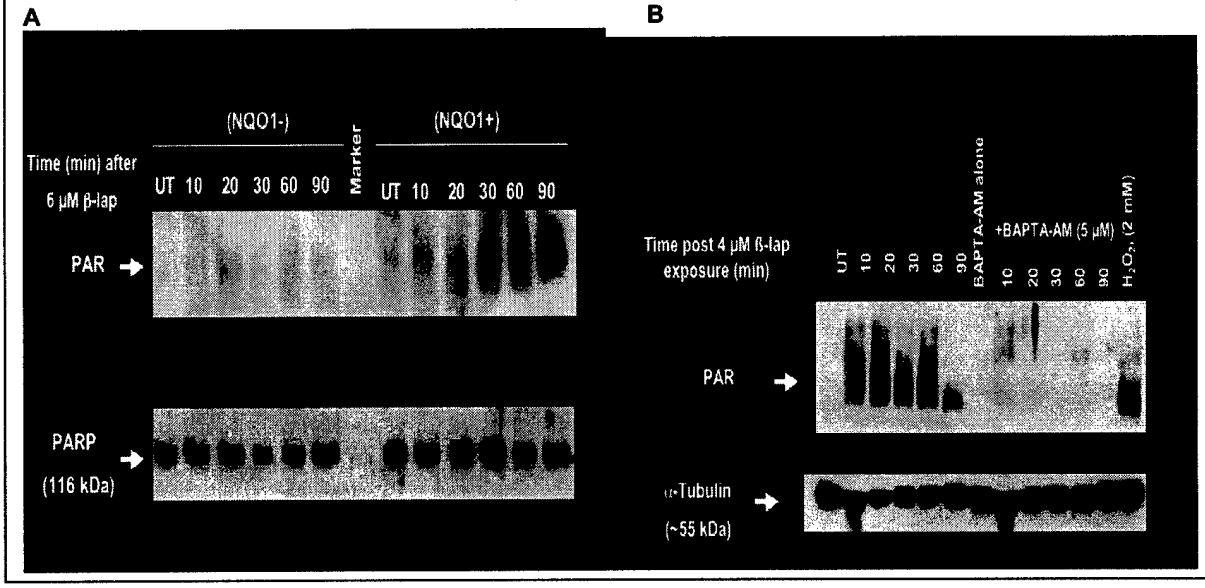
Figure 2. **NQO1 expression is required for radiosensitization of LNCaP cells by ß-lap.** $NQO1^-$ LNCaP pcDNA3 vector alone and LNCaP $NQO1^+$ clone 2 (NQCl2) cells were exposed to various IR doses, followed by various concentrations (in µM, 4-h) of ß-lap. Changes in colony forming ability. In A, pcDNA3-vector alone cells were tested. In B, same as A, except that $NQO1^+$ LNCaP cells were used. At 2 Gy, ß-lap at all doses in combination with IR resulted in statistically greater ($p<0.05$) lethality than IR alone.



TASK 2. Establish repressor-expressing (either ecdysone- or tetracycline-inducible repressor), stably (neo resistant) transfected LNCaP cells. Isolate and test clones using doxycycline- or ecdysone-responsive luciferase reporter transfection assays ***(Months 1-4)***. Completed.

Progress: We previously developed tetracycline-inducible LNCaP cells that induce TRE-luciferase >50-fold after various doses of tetracycline. We then constructed a pTRE-NQ01-neo plasmid for transfection and isolated clones that conditionally induced NQ01 via administration of tetracycline. We isolated one clone that demonstrated a 3-fold induction of NQO1 levels, with corresponding enzymatic activities noted after 1-10 μM doxycycline administration. Unfortunately, we also noted that the system was leaky in all clones tested, and that NQO1 activity was noted even in TRE-NQO1 LNCaP cells without doxycycline administration. In testing the radiosensitization of TRE-NQO1 expressing LNCaP cells with or without doxycycline we unexpectedly found that both doxycycline- or untreated TRE-NQO1-LNCaP cells were radiosensitized by ß-lapachone, as found in Fig. 2 with cells constitutively expressing NQO1. In contrast, LNCaP cells containing vector alone were not radiosensitized by ß-lapachone. These data are consistent with data shown in Figure 2, and reinforce our conclusion that NQO1 levels are **required** for radiosensitization. Inducible levels of the enzyme are, therefore, sufficient for radiosensitization, but induction is not required if the enzyme is already expressed.

Figure 3. **In A,** exposure of NQO1+, but not NQO1-, cells results in PAR formation. LNCaP cells expressing or lacking NQO1 levels were exposed to 4 µM ß-lap for 2 h. PAR formation was monitored. Blots represent experiments performed three times. **In B,** NQO1+ LNCaP, DU145, or PC-3 cells were pre-treated with BAPTA-AM (5 mM) for 1 h. Cells were then treated with ß-lap (4 µM, 4h), with or without 5 mM BAPTA-AM. Cells were then extracted at various times and whole cell extracts analyzed for poly(ADP-ribose) polymers. Shown are DU-145 cells. **In A and B,** blots represent experiments performed at least three times.



Task 3. NQO1 expressing clones (LNCaP, DU-145, or PC-3) will be treated with ß-lapachone or menadione in the presence or absence of dicoumarol, or other NQ01 inhibitors. NQ01 protein and enzyme levels will then be monitored. ***(Months 2-4).*** **Completed.**

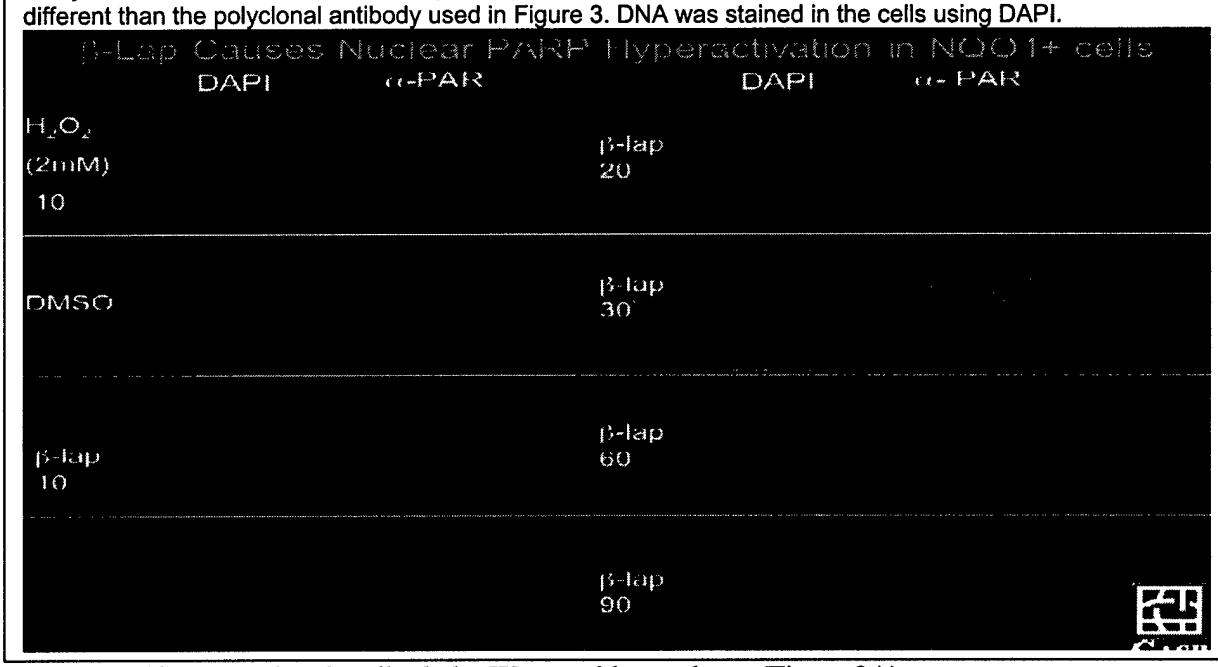
Progress: Dicoumarol administration prevented ß-lapachone lethality and radiosensitization, consistent with the data of **Figure 2.**

Task 4. Cells expressing or lacking NQO1 will then be treated with IR, +/-ß-lapachone, +/-gradient doses of dicoumarol, or +/- gradient doses of BAPTA-AM for 4h post-IR; BAPTA-AM is a calcium chelator that indirectly prevents ß-lapachone toxicity by preventing calpain or a calpain-like protease from being activated. Dicoumarol inhibits NQ01 and prevents ß-lapachone activation. IR responses will be compared to drug alone. Survival, apoptosis (caspase activation, TUNEL, apoptotic death substrate cleavage events, and pRb dephosphorylation assays), and alterations in cell cycle checkpoints will be examined; no alterations in checkpoints have yet been noted with this compound, but this information will be gathered by flow cytometry when examining apoptosis +/-TUNEL staining ***(Months 4-12). Completed.***

Progress: These studies have been completed and we discovered that ß-lap induces a calcium-dependent PARP-1 hyperactivation response, resulting in apoptosis inducing factor (AIF). Our results demonstrate that:

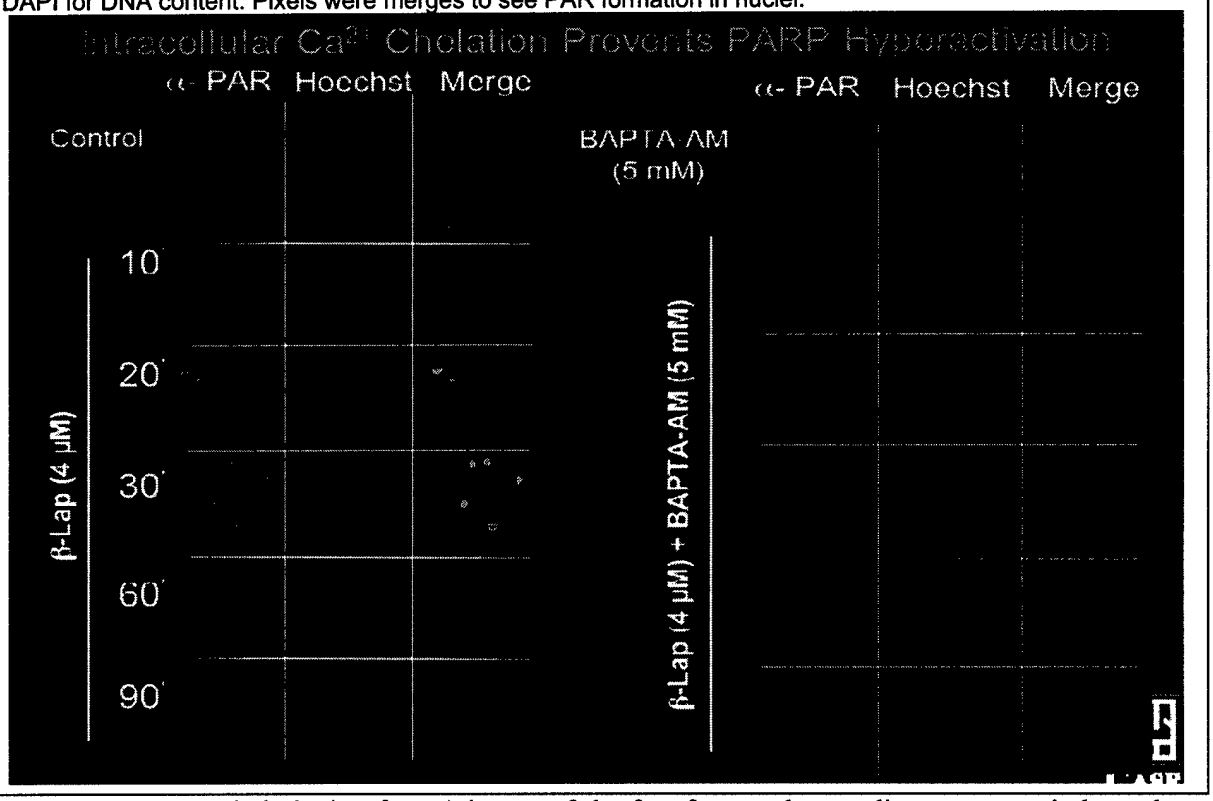
1. The previously noted loss of NAD+ levels after ß-lap exposure prompted us to examine NQO1-expressing cells treated with ß-lap for the hyperactivation of PARP-1, as noted by the elevated formation of poly(ADP-ribose) (PAR) moieties monitored by detection using a specific monoclonal antibody by Western blot analyses (Figure 3A).
2. Co-administration of BAPTA-AM prevented PAR formation induced by ß-lap as monitored by Western blot analyses (Figure 3B).

Figure 4. NQO1+ LNCaP cells were treated as in Figure 3 and monitored for PAR formation using confocal analyses and a monoclonal antibody recognizing poly(ADP-ribose) polymers. The PAR antibody was different than the polyclonal antibody used in Figure 3. DNA was stained in the cells using DAPI.



3. PARP-1 hyperactivation was confirmed by confocal analyses showing enhanced PAR formation in NQO1+ cells (Figure 4). We also noted ß-lap-induced H2AX phosphorylation specifically in NQO1+ cells, indicating that ß-lap may cause DNA damage (Bentle in prep.,).
4. ß-Lap-mediated PAR formation and increased H2AX phosphorylation are calcium-dependent, wherein BAPTA-AM prevented PAR formation (Figure 5B) and H2AX-Phosphorylation (not shown). We conclude that Ca2+ of the nucleus may activate a calcium-dependent endonuclease that triggers ß-lap-induced PARP hyperactivation and cell death.

Figure 5. NQO1+ DU-145 cells were pre-treated with 0.01% DMSO or BAPTA-AM in 0.01% DMSO (5 mM, 1 h), then exposed to 4 μM ß-lap. Cells were then stained for PAR formation and and nuclei stained with DAPI for DNA content. Pixels were merges to see PAR formation in nuclei.

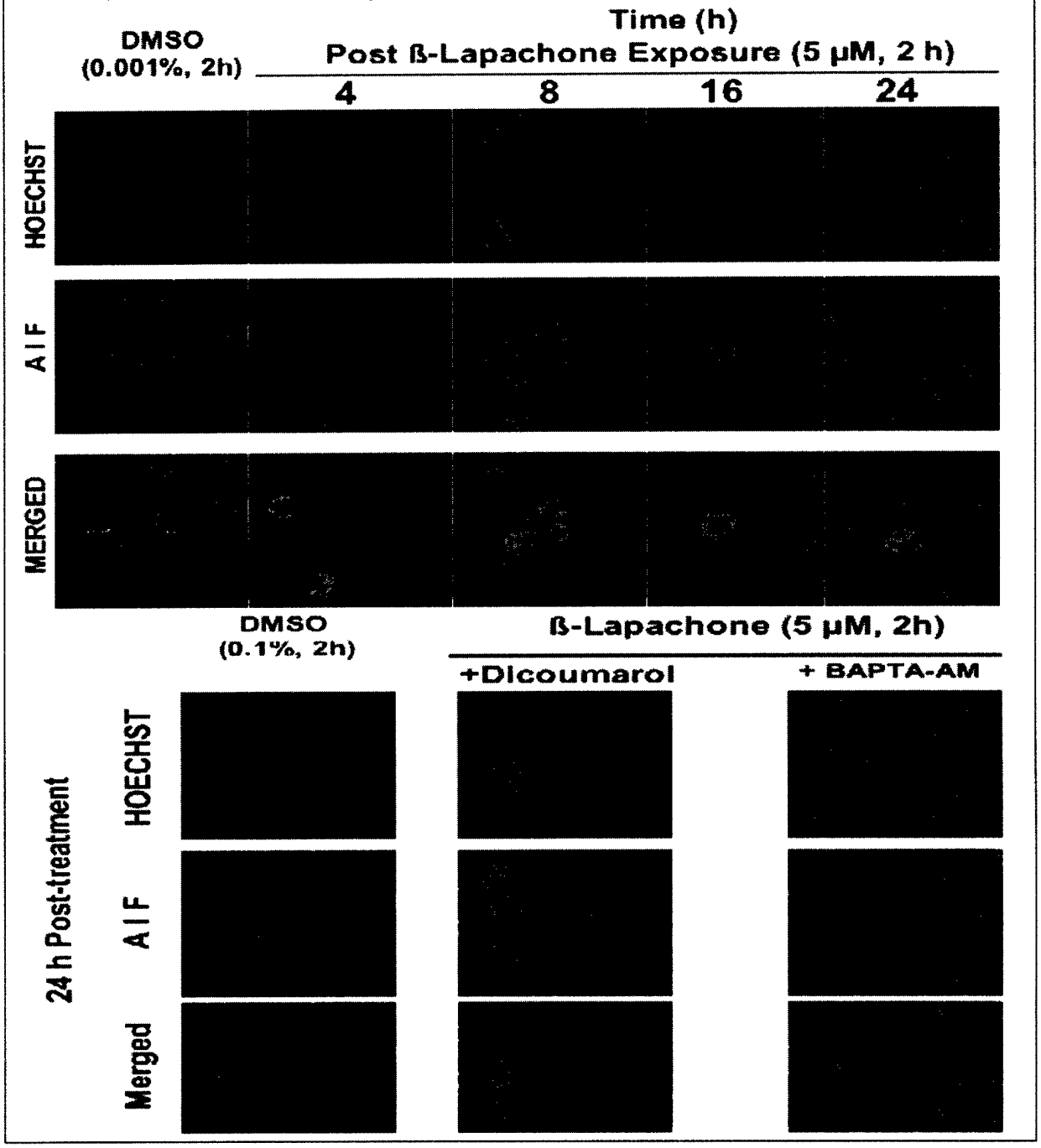


5. AIF (apoptosis inducing factor) is one of the few factors that mediates caspase-independent apoptotic reactions in cells. Since ß-lap causes apoptosis independent of caspases, and usually accompanies PARP hyperactivation, we examined the release of this factor from the mitochondria. Interestingly, ß-lap induced AIF activation in a calcium-dependent (blocked by BAPTA-AM) and NQO1-specific manner, which was blocked by dicoumarol (40 μM) (Figure 6) and not observed in NQO1- cells.

Task 5. Menadione treatments will then be compared to ß-lapachone. Responses for the two drugs should be opposite. ***(Months 4-12). Menadione exposures were completed, and opposite results were found with respect to NQO1 expression. Menadione also induced Ca2+ release, PAR formation and AIF release, however, NQO1 enhanced the responses, whereas NQO1+ cells were more resistant to this drug. These results are consistent with our prior lethality responses of this drug compared to ß-lap in NQO1+ vs. NQO1- cells (Pink et al., JBC, 2000).***

Task 6. U1-Mel cells, a malignant melanoma cell line known to induce NQ01 after IR, will be investigated as a positive control; we originally cloned NQ01 as x-ray-inducible protein #3 (xip3)

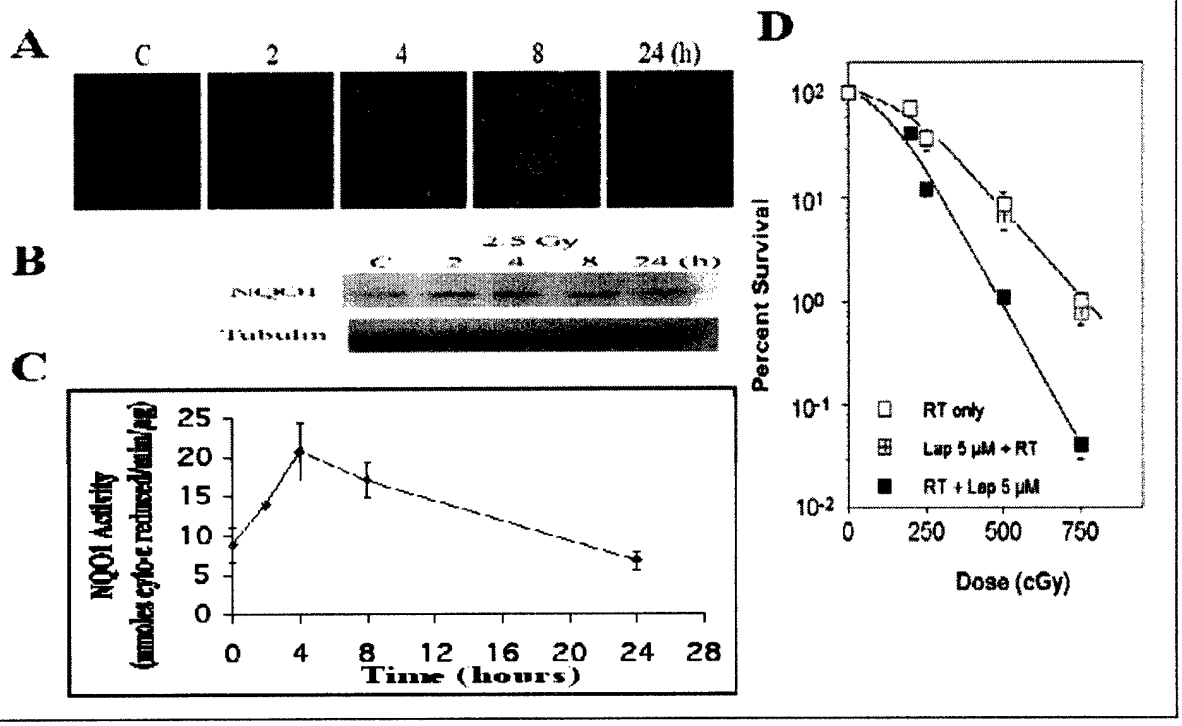
Figure 6. **Top,** ß-Lap-treated cells were examined for AIF release from mitochondria in a time-course manner. Note the loss of AIF from the mitochondria in 4h, and significant amount of AIF in the nuclei of treated cells at 8 h after exposure. Bottom, BAPTA-Am treatment as in Figures 3 and 5 prevented AIF activation, cell death, and inhibited long term clonogenic lethality caused by ß-lap.



from these cells and followed the induction kinetics of NQ01 transcript levels after IR exposure. ***(Months 0-12).***

Progress: Studies using U1-Mel and FSA squamous cell carcinoma cells have been completed, purely as 'proof of principle' that inducible NQO1 levels were sufficient for ß-lapachone-mediated radiosensitization. These studies were completed in these non-prostate cancer cell lines because we have been unable to identify a human prostate cancer cell line that expresses low levels of NQO1. Aside from LNCaP cell lines that have no detectable NQO1 levels or enzymatic activities, we have not been able to find a human CaP cell line with low levels of this two-electron reductase. Interestingly, we have also found multiple LNCaP cell lines that express elevated endogenous levels of NQO1, suggesting a large variability of NQO1 expression in LNCaP cells. Using U1-Mel or FSA cells, we found that NQO1 levels and activities were IR-inducible and that only after IR-induction was radiosensitization of cells by ß-lapachone noted.

Figure 7. **NQO1 is up-regulated by IR, and cells subsequently radiosensitized.** FSA (shown) or U1-Mel cells were exposed to IR and NQO1 levels monitored by confocal (A), western blot dB), and standard NQO1 enzyme activity analyses (D). Note that ß-lapachone only radiosensitized FSA cells when the drug was administered after IR and not before. Identical results were found using U1-Mel cells.



Task 7. Evaluation of the role of NQ01 in the survival of CaP cells following IR independent of ß-lapachone exposures. ***(Months 4-12).***

Progress: We have completed this task as discussed above. Expression of elevated levels of NQO1 in LNCaP cells offered no radioprotection or radiosensitization to cells compared to NQO1⁻ LNCaP vector alone containing cells.

Aim 2: Investigate the role of NQ01 in ß-lapachone-mediated radiosensitization *in vivo*.

Establish xenografts in male nude mice containing androgen-slow release capsules from transfected as well as non-transfected LNCaP cell lines expressing constitutive (DU-145, PC-3, LNCaP-NQ clones (Fig. 2), or conditionally-inducible (doxycycline- or ecdysone-responsive) NQ01 levels. ***(Months 0-24).***

Progress: We have been able to successfully grow DU-145 and PC-3 xenografts in male athymic nude mice without addition of androgen capsules. We also demonstrated that male athymic nude mice can support the growth of LNCaP xenografts, either expressing or lacking NQO1. In addition, the relative growth rates of LNCaP xenografts expressing or lacking NQO1 were identical. Western blot analyses of $NQO1^+$ LNCaP xenografts at t=o prior to subcutaneous injection of cells from culture, and xenografts formed at 70 days after growth in the athymic animals, showed that on a per protein basis no loss or change of NQO1 levels were noted. Enzymatic assays confirmed this result. Thus, NQO1 expression was stable in xenografts formed from NQO1-expressing LNCaP cells derived from culture.

Our progress on Aim 2 was focused on developing delivery systems for ß-lapachone and beginning our first drug delivery of ß-lapachone into mice, both C57blk/6 test mice and then male athymic nude mice. Simultaneously, we completed our first animal studies, and grew LNCaP, DU-145 and PC-3 xenografts in male athymic nude mice.

We explored various treatment schedules with ß-lapachone complexed in HP-ß-CD for enhanced solubility and improved bioavailability. In Figure 8, we demonstrate a summary of our antitumor results using this solubilized ß-lapachone preparation. Experiments were initiated by injecting male athymic nude mice with 1 X 10^6 PC-3 or DU-145 cells subcutaneously. Tumors were allowed to grow to a volume of 20-30 mm^3 before animals were injected with various doses of ß-lapachone complexed and solubilized with HP-ß-CD. Experiments were performed three times and means +SE are graphed.

Athymic male nude mice (18-20 gms) exposed to 65-75 mg/kg ß-lapachone complexed in HP-ß-CD showed significant antitumor activity alone (minimum $p<0.01$ found if 70 mg/kg ß-lapachone in HP-ß-CD was injected). The maximum tolerated dose of ß-lapachone, when given as 10 injections once per day for 10 days, was ~90 mg/kg. Doses of ß-lapachone below 65 mg/kg under this dose treatment schedule showed minimal antitumor activity. Near identical results were observed in female athymic mice bearing $NQO1^+$ MDA-MB-468 or MDA-MB-231 breast cancer xenografts.

In the final year of this grant we: **(a)** explored different dose regimen of ß-lapachone using the same tumor models; **(b)** optimized the antitumor activity of ß-lapachone alone by altering treatment regimen scheduling; and **(c)** explored the antitumor activity of ß-lapachone in combination with various doses of external beam IR.

We discovered that ß-lap given in HPß-CD was too toxic. We then discovered that ß-lap administered in PLGA-PEG millirods could be administered in a controlled fashion with glucose concentrations. We then developed millirods that could deliver several bolus doses of ß-lap by

making 'ringed' millirods wherein a ß-lap containing PLGA-PEG ring was covered in a ring with no ß-lap. Alternating rings are made to give multiple pulses of ß-lap.

We are continuing the IR synergy studies, but have been having trouble growing LNCaP xenografts that express or are deficient in NQO1. The results in Figure 8 were encouraging, however, we realize that the treatment schedule (1X/day every other day) is not optimal and does not fit the mechanism of ß-lapachone. ß-Lapachone works irreversibly to kill cells after exposure to the drug for a minimum of 2 h. Thus, treatment regimen that lead to heightened bolus doses/concentrations of drug in the blood would be optimal. Therefore, we are now experimenting with 2 doses of drug per day. We have already determined the toxicology of this regimen and found that animals tolerate this split dosing better. For example, we have found that the MTD is 50 mg/kg given 2X/day for 5 consecutive days. Given that the MTD of the schedule used in Figure 4 was 90 mg/kg, athymic nude mice appear to tolerate higher overall doses of the drug in a shorter period of time. Interestingly, we also noted significant antitumor activity in these mice at a minimum dose of 30 mg/kg given 2X/day for 5

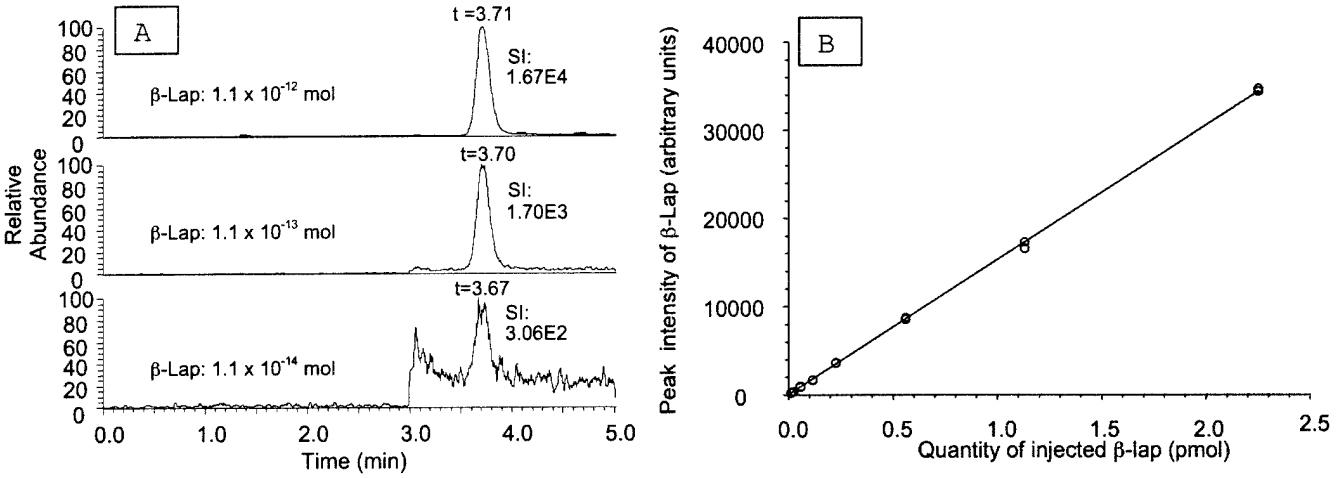


Figure 9. Analyses of ß-lap by HPLC-ESI-MS. **(A)** Chromatograms of β-lap by HPLC-ESI-MS analyses. Injected quantities of β-lap are shown above each chromatogram. Signal Intensities (SI) of peak values are stated for each chromatogram. The bottom chromatogram shows the sensitivity limit of quantification at 1.1 x 10^{-14} mol of injected β-lap (signal:noise ~4:1). **(B)** Correlation of peak intensity in HPLC-ESI-MS analyses with known quantities of β-lap (duplicate samples were used). Fig. 12B shows a calibration curve for future quantitative analyses of β-lap in Aim #3.

consecutive days. The injected mice were control mice containing huge tumor masses (>5 cm^3) and showed significant antitumor activities at the end of the injections. These studies, with or without IR, are being completed using millirods that can be used in conjunction with radioactive seeds as used in brachytherapy.

Quantitative analyses of β-lap by HPLC-ESI-MS. Finally, we were interested in developing electrospray-mass spectrometry-High Pressure Liquid Chromatography methodology for the analyses of ß-lapachone levels in the blood, tumor, and normal tissues. To evaluate the *in vivo* pharmacokinetics of β-lap delivery from polymer microspheres, it was necessary to develop an accurate, highly sensitive, and quantitative method to measure the plasma concentration-time relationships and tissue distribution of β-lap. We developed high pressure liquid chromatography-electrospray ionization-mass spectrometry (HPLC-ESI-MS) methodology for the quantitative

analyses of β-lap that represents a modification and dramatically increased sensitivity of β-lap detection over previous techniques (*117*). Fig. 9A shows the chromatograms of β-lap at three different injected quantities. The chromatographic medium was Ansys Metachem Polaris C18A (3 μm particle diameter) contained inside a column of 0.46 cm i.d. x 5 cm in length. The isocratic mobile phase was 25 mM ammonium formate + acetonitrile (v/v = 50/50), at a flow rate of 0.5 mL/min. β-Lap was detected by selective reaction monitoring of the transition from 243 m/z $(M+H)^+$ to 187 m/z. Preliminary data showed superb sensitivity of detection under current experimental conditions. The lowest limit of quantification was 1.1 x 10^{-14} mol of injected β-lap with a signal to noise ratio of 4:1 (Fig. 9A, bottom). For a biological sample volume of 100 μL, this sensitivity permits detection of β-lap at 0.1 nM (10^{-10} M). Fig. 9B shows a calibration curve between peak intensity in HPLC-ESI-MS analyses and known quantities of β-lap. Detector response was linear over a concentration range of 2.3 x 10^{-9} M to 4.5 x 10^{-6} M of β-lap (r^2 = 0.999). This calibration curve provides the basis for future quantitative analyses of β-lap levels *in vivo*. These data show the feasibility of HPLC-ESI-MS for highly sensitive and quantitative analyses for β-lap, as outlined in Aims #2 and #3.

Summary: In summary, we completed most of the tasks outlined in Aims 1-3. We are focusing on Aim 3. We have discovered that ß-lapachone radiosensitizes cells provided that NQO1 activity is expressed. Cells deficient in NQO1 expression are not radiosensitized. We also found that over-expression of NQO1 does not confer radioprotection or radiosensitivity to human prostate cancer cells, since the clonogenic survival responses of $NQO1^+$ v. $NQO1^-$ human CaP cells were not significantly different.

Mechanistically, we discovered that ß-lap causes a rapid ER Ca2+ release, and a subsequent loss of NAD+ and ATP levels within 30 mins of exposure. A minimum of 2h exposure to 4 μM ß-lap is required for lethality. Ca2+ release is essential for ß-lap lethality, and co-administration of BAPTA-AM completely prevented lethality. Loss of NAD+ appears to be caused by PARP hyperactivation, which appears to be Ca2+-dependent. Release of Ca2+, loss of NAD+ somehow causes release of AIF from the mitochondria. We hypothesize that AIF release leads to ß-lap-mediated, calpain-dependent, caspase-independent apoptosis. This hypothesis is being explored currently in a submitted NIH grant using CaP as well as breast cancer cell lines expressing or lacking NQO1.

We also made significant progress on elucidating the antitumor activity of ß-lapachone alone in Aim 3 and our current studies remain focused on determining the optimal scheduling of ß-lapachone based on the compound's mechanism of action. For example, preliminary data strongly suggest that injection of male athymic nude mice bearing $NQO1^+$ PC-3 xenografts (animals bearing >10 cm^3 xenografts) with two doses of 20-40 mg/kg ß-lapachone (with an 8-h interval between injections), caused significant regression of tumor mass. The ß-lapachone injection doses were well tolerated with minimal weight loss, and these injection schedules, as well as many others, are now being evaluated for toxicity and antitumor efficacy. Finally, the development of a reliable and sensitive **HPLC-ESI-MS** method for analyzing ß-lapachone levels in blood and normal and tumor tissues should allow us to monitor active drug levels in the animals over time and correlate antitumor responses or normal tissue toxicity with various efficacious v. non-efficacious treatment regimen of ß-lapachone in athymic nude mice bearing human prostate xenografts.

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

We have accomplished most of the Aims of this grant and have developed a novel millirod delivery system for exposing human prostate cancers with ß-lap during brachytherapy. This methodology is currently being explored in a newly funded DOD CaP grant. We have determined/generated that:

Aim #1:

1. NQ01-expressing LNCaP cells are sensitive to ß-lapachone (ß-lap).
2. As expected, Dicoumarol suppressed ß-lap-induced cell death (apoptosis).
3. LNCaP cells are able to conditionally induce NQO1 in response to exogenously administered tetracycline.
4. NQO1 expression is required for radiosensitization by ß-lapachone.
5. Cells deficient in NQO1 expression are not radiosensititized by ß-lapachone.
6. Induction of NQO1 after IR in U1-Mel and FSA cells is sufficient for radiosensitization by ß-lapachone.
7. The apoptotic responses (morphology changes, atypical PARP and p53 proteolytic events) noted in $NQO1^+$ LNCaP cells exposed to 5 µM ß-lap were equivalent to responses observed in IR-treated $NQO1^+$ LNCaP exposed to 2-3 µM ß-lapachone, 2-4 h. In contrast, exposure of the same cells to 2-3 µM ß-lapachone for 2-4 h did not cause a significant cytotoxic response.

Aim #2:

8. LNCaP cells with conditionally inducible (via doxycycline) NQO1 levels were developed, and tested for radiosensitization responses. All clones were leaky for NQO1 and were radiosensitized by ß-lapachone. In contrast, vector alone LNCaP cells were not radiosensitized by ß-lapachone.
9. ß-Lap exposure causes ER Ca2+ release.
10. ß-Lap exposure causes hyperactivation of PARP, resulting in loss of NAD+ levels, as well as dramatic ATP loss.
11. ß-Lap exposure causes the release of AIF and its translocation into the nuclei of exposed cells correlates with nuclear condensation and TUNEL+ staining (i.e., apoptosis).
12. LNCaP, PC-3 and DU-145 cells were successfully grown as xenografts in male athymic nude mice.
13. The solubility and bioavailability of ß-lapachone were significantly increased by the use of ß-cyclodextrin or HP-ß-CD.
14. Several additional potential routes of drug delivery have been developed, including millirods and microspheres. The drug is trapped in biodegradable, biopolymers with glucose and drug release can be controlled. The millirods will be used for brachytherapy, the microspheres for systemic or aerosol drug delivery.
15. ß-Lapachone, solubilized in HP-ß-CD, was well tolerated in male athymic nude mice up to ~100 mg/kg.
16. ß-Lapachone administered at 65-75 mg/kg showed significant antitumor activity in athymic mice bearing $NQO1^+$ PC-3 or DU-145 xenografts. Furthermore, NQO1 expression was maintained in

LNCaP xenografts and NQO1-defifient cells were not selected for as LNCaP xenografts grew. Studies on the efficacy of ß-lapachone using $NQO1^+$ v. $NQO1^-$ LNCaP xenografts are now ongoing.

17. Finally, we have developed **HPLC-ESI-MS** methodology for the quantification of ß-lapachone levels in the blood and tissues of mice and we will use this methodology to evaluate levels of the parent drug after various treatment schedules of ß-lapachone.
18. After optimizing ß-lapachone treatment schedules, we will investigate the antitumor efficacy of ß-lapachone with or without external beam exposures, and evaluate ß-lapachone blood and tissue levels to use as laboratory correlates to predict drug efficacy.
19. Finally, we have discovered a series of ß-lap prodrugs that are activated in NQO1+ cells by Schiff's base reactions (see Reinicke et al., JBC, submitted, 2004).

Thus, we found that ß-lapachone was efficacious alone against human prostate cancer cells expressing NQO1. Further studies to be performed will examine the efficacy of ß-lapachone after various treatment schedules. Finally, combination treatments of ß-lapachone with external beam IR will be explored. A grant to the DOD Prostate Cancer Initiative Program was funded to explore the potential efficacy of ß-lapachone and brachytherapy using PLGA polymer millirod release kinetics of this drug. An NIH grant to examine the roles of Ca2+ release, PARP hyperactivation, and AIF release/translocation was submitted, received a score of 1.8 (25%) and was resubmitted after revision to fit the first review. We are hopeful of its funding.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes to include:

CURRENT LIST OF PUBLICATIONS RESULTING FROM THIS AWARD

PAPERS PUBLISHED IN PEER-REVIEWED JOURNALS (Enclosed where applicable):

-Tagliarino, C., Pink, J.J., Dubyak, G.R., Nieminen, A-L., and Boothman, D.A. Calcium is a key signaling molecule in ß-lapachone-mediated cell death. 2001; J. Biol. Chem. 276(22): 19150-19159. *(Previously submitted).*

-Planchon, S.M., Pink, J.J., Tagliarino,, C., Bornmann, W.G., Varnes, M.E., and Boothman, D.A. ß-Lapachone-induced apoptosis in human prostate cancer cells: involvement of NQO1/xip3. 2001; Exp. Cell Res., 267: 95-106. *(Previously submitted).*

-Tagliarino, C. Pink, J.J. and Boothman, D.A. Calpains and apoptosis. 2001; Korean J. Biol Sci., 5: 267-274. *(Previously submitted).*

-Tagliarino, C., Pink, J.J., Reinicke, K., Simmers, S.A., Wuerzberger-Davis, S., and Boothman, D.A. Cancer Biology and Therapy, 2 (2): 154-162, 2003. *(Enclosed).*

-Nasongkla, N., Wiedmann, A., Bruening, A., Beman, M., Ray' D., Bornmann, W.G., Boothman, D.A. and Jinming Gao. Enhancement of Solubility and Bioavailability of b-Lapachone Using Cyclodextrin Inclusion Complexes. J. Pharm. Research, 2003.

-Ahn, K-J, Song, C-W, Choi, Chi, EI, Williams, B, Griffin, R, Bey, E, Gao, JM, Bornmann, WG, Boothman, DA, Park, HJ. Radiation increases the sensitivity of cancer cells to ß-lapachone. Cancer Res., *In Press*, 2003.

--Reinicke, K, Bey, E, Pink, JJ, Bentle, M, Nasongkla, N, Burton, G, Gao, J and Boothman, DA. Generation and development of ß-lapachone prodrugs. J. Biol. Chem., *Submitted,* 2004 (enclosed).

PAPERS PUBLISHED IN NON-PEER-REVIEWED JOURNALS (Not Enclosed).

***Abstracts and Presentations Related to this Grant:**

Several presentations using data generated from this DOD grant proposal were give during the previous funding period. These are listed below:

1. ß-Lapachone, a kiss of death. Case Western Reserve University, Blood Club., February, 2002.
2. ß-Lapachone, a kiss of death. University of South Florida-Tampa, January, 2003.
3. NQO1-mediated cell death by ß-lapachone. NIH Workshop on low dose IR effects. November, 2002.
4. Exploiting stress. DOE/NIH Workshop, November, 2002.

Patents and licenses applied for and/or issued.

-We filed a patent through CWRU on the use of hydroxypropyl-ß-cyclodextrin (HP-ß-CD), as well as PLGA polymer nanoparticles, microspheres, and millirods for the delivery of ß-lapachone *in vivo*. The title of this now submitted patent is: ß-Lapachone Delivery Systems, Compositions and Uses Related Thereto. Authors are Jinming Gao; David A. Boothman and John J. Pink.

Degrees Obtained During This Award.

1. Tagliarino, Colleen, Ph.D., Case Western Reserve University, Dept. Pharmacology, October, 2001.
2. Planchon, Sarah, Ph.D. University of Wisconsin, Dept. Human Oncology, November, 2000.
3. Kate Reinicke, expected graduation Dec., 2005. Ms Reinicke was selected as the Case representative to attend the 41st Annual Meeting of Nobel Laureates in June of 2003. She was selected in early June of last year to attend the meeting, wherein only 40 students were selected throughout the Nation.
4. Melissa Bentle, expected graduation, May 2006. Ms Bentle successfully competed for a DOD pre-doctoral fellowship to examine ß-lap-mediated cell death responses in breast cancer cells. Additionally, Ms. Bentle successfully competed for the spot at Case to attend the 42nd Annual meeting of Nobel Laureates. Last week she was also selected to attend the meeting in June of this year.

Development of Cell Lines, Tissue or Serum Repositories:

-LNCaP NQ CLONES 1-10: human NQ01-deficient cells stably transfected with CMV-directed NQ01.
-vector-alone LNCaP cells.
-Tetracycline-inducible, LNCaP cells.

Informatics such as databases and animal models, etc.: None

Funding applied for based on work supported by this award. :

-A complementary grant application to explore the use of PLGA millirods carrying ß-lapachone in combination with ^{192}Ir-low dose rate radioactive seeds has been funded recently by the DOD. The information regarding this grant application is listed below:

Use of ß-lapachone-encapsulated millirods for improved therapy of prostate cancer. DOD funded 04/01/04, David A. Boothman, Ph.D., PI.

Employment or research opportunities applied for and/or received on experiences/training supported by this award.

-Colleen Tagliarino, Ph.D. Post-doctoral Fellow, Johnson and Johnson Drug Development Center, Philadelphia, PA.
-Colleen Tagliarino, Ph.D.Assistant Researcher, Aventis Pharmaceuticals Prostate and Breast Cancer Research Division.
-Sarah Planchon, Ph.D. Post-doctoral fellow, Cleveland Clinic Foundation.

CONCLUSIONS: Summarize the results to include the importance and/or implications of the completed research and when necessary, recommend changes on future work to better address the problem. A "so what section" which evaluates the knowledge as a scientific or medical product shall also be included in the conclusion of the annual and final reports.

We have made a number of important advances in the three years of this grant. We found that:

1. ß-Lapachone can radiosensitized NQO1-expressing, but not NQO1-deficient prostate cancer cells.
2. Expression of NQO1 does not confer protection or sensitization to IR.
3. IR-induction of NQO1 levels and activities is sufficient for radiosensitization by ß-lapachone.
4. 4. ß-Lap induces a Ca2+ release from the ER, hyperactivated PARP, NAD+ and ATP levels dramatically drop, and AIF is released from the mitochondria. AIF release causes caspase-mediated cell death, and possibly results, of involves in the activation of μ-calpain.
5. ß-Lapachone, when solubilized in HP-ß-CD demonstrated significant antitumor activity against NQO1+ CaP xenografts when grown in male athymic nude mice.
6. We developed a reliable and sensitive electrospray HPLC method for evaluating ß-lapachone levels in blood and tissues.
7. We developed ß-lap-encapsulated millirods.
8. ß-Lap radiosensitizes NQO1+ CaP tumors, as well as U1-Mel and FAS cells. Additional research on the mechanisms of ß-lap radiosensitization is warranted.

REFERENCES: List all references pertinent to the report using a standard journal format such as *Science, Military Medicine,* etc.:

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APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples of appendices include journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Prior Appendix Items Already Supplied to the DOD:

-Planchon, S.M., Pink, J.J., Tagliarino,, C., Bornmann, W.G., Varnes, M.E., and Boothman, D.A. ß-Lapachone-induced apoptosis in human prostate cancer cells: involvement of NQO1/xip3. Exp. Cell Res., 267: 95-106, 2001.

-Tagliarino, C., Pink, J.J., Dubyak, G.R., Nieminen, A-L., and Boothman, D.A. Calcium is a key signaling molecule in ß-lapachone-mediated cell death. 2001; J. Biol. Chem. 276(22): 19150-19159.

-Planchon, S.M., Pink, J.J., Tagliarino,, C., Bornmann, W.G., Varnes, M.E., and Boothman, D.A. ß-Lapachone-induced apoptosis in human prostate cancer cells: involvement of NQO1/xip3. 2001; Exp. Cell Res., 267: 95-106.

-Tagliarino, C. Pink, J.J. and Boothman, D.A. Calpains and apoptosis. 2001; Korean J. Biol Sci., 5: 267-274.

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Tagliarino, C. Pink, J.J. and Boothman, D.A. Calpains and apoptosis. Korean J. Biol Sci., 5: 267-274, 2001.

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Research Paper

Enhancement of Solubility and Bioavailability of β-Lapachone Using Cyclodextrin Inclusion Complexes

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Purpose. To explore the use of cyclodextrins (CD) to form inclusion complexes with β-lapachone (β-lap) to overcome solubility and bioavailability problems previously noted with this drug.

Methods. Inclusion complexes between β-lap and four cyclodextrins (α-, β-, γ-, and HPβ-CD) in aqueous solution were investigated by phase solubility studies, fluorescence, and ^{1}H-NMR spectroscopy. Biologic activity and bioavailability of β-lap inclusion complexes were investigated by *in vitro* cytotoxicity studies with MCF-7 cells and by *in vivo* lethality studies with C57Blk/6 mice (18–20 g).

Results. Phase solubility studies showed that β-lap solubility increased in a linear fashion as a function of α-, β-, or HPβ-CD concentrations but not γ-CD. Maximum solubility of β-lap was achieved at 16.0 mg/ml or 66.0 mM with HPβ-CD. Fluorescence and ^{1}H-NMR spectroscopy proved the formation of 1:1 inclusion complexes between β-CD and HPβ-CD with β-lap. Cytotoxicity assays with MCF-7 cells showed similar biologic activities of β-lap in β-CD or HPβ-CD inclusion complexes (TD_{50} = 2.1 μM). Animal studies in mice showed that the LD_{50} value of β-lap in an HPβ-CD inclusion complex is between 50 and 60 mg/kg.

Conclusions. Complexation of β-lap with HPβ-CD offers a major improvement in drug solubility and bioavailability.

KEY WORDS: β-lapachone; cyclodextrin; inclusion complex; solubility; bioavailability.

INTRODUCTION

β-Lapachone (β-lap) is a potent cytotoxic agent that demonstrates antitumor activity against a variety of human cancer cells. The drug was first isolated from the bark of the *Lapacho* tree (genus *Tabebuia*) in the rainforests of South America and has a long history as an herbal medicine. β-Lap is bioactivated by the enzyme, NQO1 [NAD(P)H:quinone oxidoreductase, E.C. 1.6.99.2], which is a ubiquitous flavoprotein found in most eukaryotic cells. This enzyme catalyzes a two-electron reduction of various quinones, utilizing either NADH or NADPH as electron donor. The human NQO1 gene encodes a 30-kd protein that is expressed in a tissue-dependent manner. More importantly, NQO1 is overexpressed (up to 20-fold) in a number of tumors, including breast, colon, and lung cancers, compared with adjacent normal tissue (1–4). Overexpression of NQO1 in cancerous cells makes it an ideal target for tumor-selective drug therapies with minimal toxicities to healthy cells. Despite the potency and selectivity of β-lap in killing NQO1-containing cancer cells *in vitro*, the low water solubility of β-lap (0.038 mg/ml or 0.16 mM) limits its systemic administration and clinical applications *in vivo*.

Cyclodextrins (CDs) are a well-known class of host molecules that can form inclusion complexes with a variety of drugs to improve drug solubility, stability, as well as bioavailability (5–11). Cyclodextrins consist of different number of glucopyranose units that are connected by α(1,4) glycosidic linkages. The shape of these molecules is similar to a truncated cone (Fig. 1). which has a hydrophilic outer surface and a hydrophobic inner cavity. α-Cyclodextrin (α-CD) comprises six glucopyranose units, β-cyclodextrin (β-CD) seven such units, and γ-cyclodextrin (γ-CD) eight such units. Different numbers of glucopyranose units lead to different cavity sizes. The inner diameter of the hydrophobic cavity is approximately 4.7–5.3, 6.0–6.5, and 7.5–8.3 Å for α-CD, β-CD, and γ-CD, respectively (7). Hydroxypropyl-β-cyclodextrin (HPβ-CD) is a modified β-CD obtained by treating a base-solubilized solution of β-CD with propylene oxide. This chemical modification significantly increases the solubility of HPβ-CD over β-CD (Table I). In addition, HPβ-CD is well tolerated and appears to be safe in clinical trials without observable renal toxicity as shown with β-CD (9).

The aim of this work was to explore the use of cyclodextrins to form inclusion complexes with β-lap to overcome the solubility and bioavailability problems of the drug. We hypothesize that binding of β-lap inside the hydrophobic cavity of cyclodextrins will drive the dynamic equilibrium of β-lap from solid state to the solution state (Fig. 1), thereby increasing the drug solubility. In this study, we used UV-Vis and fluorescence spectrometry to examine the effect of four types of cyclodextrins on the aqueous solubility of β-lap. The effect of β-CD and HPβ-CD on the resonance of β-lap protons and structure of the inclusion complexes were studied by ^{1}H-NMR spectroscopy. A maximal solubility of β-lap (16.0 mg/ml or 66.0 mM) was achieved with HPβ-CD, more than 400-fold increase over β-lap solubility in water. The effect of cyclodextrins on the biologic effectiveness of β-lap was investigated *in vitro* using antitumor activity assays against human MCF-7 breast cancer cells as well as *in vivo* using toxicity and weight loss measurements after intraperitoneal (i.p.) injections of β-lap inclusion complexes in C57Blk/6 mice.

MATERIALS AND METHODS

Materials

α-CD, β-CD, γ-CD, and HPβ-CD were obtained from Cyclodextrin Technologies Development, Inc. (CTD) (High Springs, FL) with >98% purity. β-Lap was synthesized following a previously reported procedure (12). Phosphate-buffered saline (PBS, pH 7.4) was purchased from Fisher Sci-

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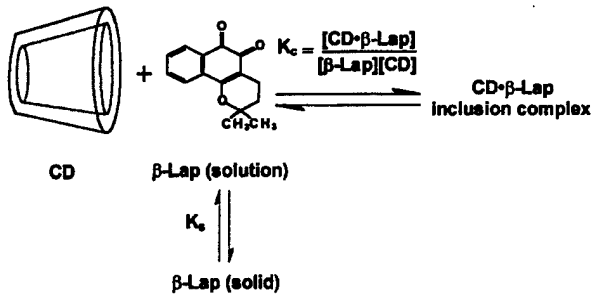

Fig. 1. Schematic diagram of the solubility equilibria of β-lap in aqueous solutions containing cyclodextrin (CD). K_s and K_c are the equilibrium constants for β-lap solubility and formation of inclusion complex, respectively. [CD·β-lap], [β-Lap], and [CD] are the concentrations of CD·β-lap complex, free β-lap, and free CD, respectively.

entific (Pittsburgh, PA). RPMI 1640 medium, fetal bovine serum, L-glutamine, penicillin, and streptomycin were purchased from Hyclone (Logan, UT) and Life Technologies, Inc. (Rockville, MD). MCF-7 breast cancer cells were routinely passed at 1:5 to 1:20 dilutions every 5 days using mycoplasma-free 0.05% trypsin as described (13).

Phase Solubility Studies of CD·β-Lap Inclusion Complexes

Solubility studies were performed by adding an excess amount of β-lap to a series of PBS buffers containing different concentrations of each CD molecule ranging from zero to its solubility limit (see Table I for the solubility limit of each CD molecule). The suspensions were stirred at 25°C until dissolution equilibrium was reached. Then aliquots were withdrawn, filtered (Nylon syringe filter, 0.2 μm pore size, from Fisher Scientific, Pittsburgh, PA), and analyzed for β-lap concentrations by UV-Vis spectrophotometry [λ_{max} = 257.2 nm, ε = 109.6 ml/(mg·cm)]. A phase solubility diagram for each CD was obtained by plotting the β-lap solubility at dissolution equilibrium as a function of the CD concentration. The association constant (K_c) for the complex formation was calculated based on Eq. (1) assuming a 1:1 ratio of complex formation (14).

$$K_c = \frac{\text{Slope}}{\text{Y} - \text{intercept} \times (1 - \text{Slope})} \quad (1)$$

^{1}H-NMR Study of CD·β-Lap Inclusion Complexes

All ^{1}H-NMR spectra were obtained on a Varian 600-MHz NMR spectrometer. The probe temperature was set at 25°C. ^{1}H-NMR spectrum of β-lap was assigned by homonuclear correlation spectoscopy (COSY) and heteronuclear multiple quantum coherence spectroscopy (HMBC). One-dimensional gradient-enhanced ROESY (GROESY) experiments were carried out by using the following pulse sequence: relaxation delay, 1 s; 90° pulse width, 8.2 μs; spin lock time, 400 ms; and acquisition time, 3.495 s. The concentrations of β-lap and HPβ-CD for the GROESY experiments were 10.6 and 58.8 mM, respectively, in D_2O.

The complex for the NMR shift titration study was prepared by adding 78 μl of β-lap stock solution (1.64 mM in MeOH). The solution was dried, and then variable amounts of β-CD and HPβ-CD solution in D_2O were added. The resulting β-lap (0.123 mM) and β-CD (0.1–14.7 mM) or HPβ-CD (0.5–430 mM) solutions were vigorously stirred at 25°C overnight to ensure the reaching of equilibrium. The association constants can be determined based on Eq. (2) (15).

$$\Delta\delta_{\text{Hc or Hd}} = \frac{K_C \Delta\delta_0 (\Delta\delta_0[CD] - \Delta\delta[\beta\text{-Lap}])}{\Delta\delta_0 + K_C (\Delta\delta_0[CD] - \Delta\delta[\beta\text{-Lap}])} \quad (2)$$

For methyl protons (Hc) on β-lap, $\Delta\delta_{Hc}$ denotes the difference of chemical shift between the two splitting methyl groups at a particular concentration of CD. For aromatic Hd protons, $\Delta\delta_{Hd}$ ($\Delta\delta_{Hd} = 7.787 - \delta_i$) is calculated as the difference between the chemical shift of pure β-lap (7.787 ppm) and that of CD·β-lap inclusion complex at a particular concentration of CD (δ_i). For both Hc and Hd protons, $\Delta\delta_0$ denotes the difference between pure β-lap and pure CD·β-lap inclusion complexes, [CD] stands for the concentration of cyclodextrin, and [β-lap] denotes the concentration of β-lap used in this experiment (0.123 mM).

Fluorescence Study of CD·β-Lap Inclusion Complexes

Fluorescence study was performed on a LS45 Luminescence Spectrometer (Perkin Elmer Instruments) with 100 nm/min scan speed and 10 nm for both excitation and emission slit widths. Initially, emission spectra of β-lap (0.015 mg/ml) in PBS buffer were obtained at different excitation wavelengths to determine the optimal values of λ_{ex} and λ_{em} for spectrophotometry measurements. The effect of CD concentrations on the fluorescence spectra of β-lap was studied. In these studies, each sample was prepared by adding the same volume (4 ml) of a stock solution of β-lap (0.005 mg/ml) but different quantities of CD inside a 5 ml volumetric flask filled with PBS buffer. The resulting solutions were vigorously stirred at 25°C overnight to ensure the reaching of equilibrium. Emission spectra of β-lap at different CD concentrations were obtained at λ_{ex} = 330 nm. The fluorescence intensity at λ_{em} = 436 nm was measured and used to determine the value of K_c of CD·β-lap inclusion complex.

In Vitro Cytotoxicity Assays

The cytotoxicity of β-CD·β-lap and HPβ-CD·β-lap inclusion complexes to MCF-7 breast cancer cells was determined

Table I. Physical Properties of α-CD, β-CD, HPβ-CD, and γ-CD (7)

Properties	Cyclodextrins			
	α-CD	β-CD	HPβ-CD	γ-CD
No. of glucose units	6	7	7	8
Molecular formula	$(C_6H_{10}O_5)_6$	$(C_6H_{10}O_5)_7$	$(C_6H_{10}O_5)_7(C_3H_6O)_{4-5}$	$(C_6H_{10}O_5)_8$
Molecular weight	972	1135	1390	1297
Cavity diameter (Å)	4.7–5.3	6.0–6.5	6.0–6.5	7.5–8.3
Solubility (mg/ml)	145	18.5	500	232

following a previously published procedure (16). The MCF-7 cells were grown in RPMI 1640 medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. In cytotoxicity studies, cells were first seeded into 96-well plates at 1×10^4 cells/well in 1 ml medium and allowed to attach overnight. Medium was removed 24 h later, and new medium (1 ml) containing different concentrations of CD alone or β-lap in CD inclusion complex was added to each well. After 4 h, the medium was removed and replaced with drug-free growth medium. Cells were allowed to grow for an additional 6 days. On day 7, cells were washed with PBS after medium removal, and 250 μl double-distilled Milli Q H_2O was added to each well. After one freeze-thaw cycle, TNE buffer (2 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4) with 10 μg/ml Hoechst 33258 (Sigma) fluorescent dye was added to each well. Changes in cell number, measured as DNA content, were then determined by an adaptation of the method of Labarca and Paigen (17) and analyzed with a Perkin Elmer HTS 7000 Bio Assay Reader with excitation wavelength of 360 nm and emission wavelength of 460 nm. Data were expressed as relative growth (T/C) by dividing DNA content of treated cells (T) by that of untreated cells (C) at identical times. The reproducibility of each data point is represented by the means ± SEM of at least six replicate wells. β-Lap in dimethylsulfoxide (DMSO) was used as a positive control to compare the drug cytotoxicity to MCF-7 cells.

Animal Toxicity Studies

C57Blk/6 female mice (3–4 weeks old, 18–20 g) (Jackson Labs, Maine) were used to study the morbidity and mortality of mice treated with HPβ-CD·β-lap inclusion complex. Four mice per group were used for each dose, which varied from 20 to 100 mg/kg. Two groups of four mice were used for 60 mg/kg because this dose proved to be near the LD50 (lethal dose that kills 50% of the mice population) of the β-lap in HPβ-CD inclusion complex. Mice were injected (i.p.) every Monday, Wednesday, and Friday for a total of 10 injections. Control animals (four mice/group) were injected with 5000 mg/kg of HPβ-CD alone to evaluate its toxicity. This HPβ-CD dose is approximately 10 times the HPβ-CD amount introduced at the highest dose of β-lap (100 mg/kg) via the HPβ-CD·β-lap inclusion complex. The higher dose of HPβ-CD was used to ensure the lack of toxicity of this compound. Weight and lethality were measured on a daily basis following initial drug administration. All animals were maintained in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care according to the "Principles of Laboratory Animal Care" of the National Institutes of Health.

RESULTS AND DISCUSSION

Solubility Study

The effect of cyclodextrins on the aqueous solubility of β-lap was evaluated using the phase solubility method (14). Fig. 2 shows the phase diagrams of β-lap with four different types of CDs in PBS buffer. The solubility of β-lap increased linearly as a function of α-, β-, or HPβ-CD concentrations.

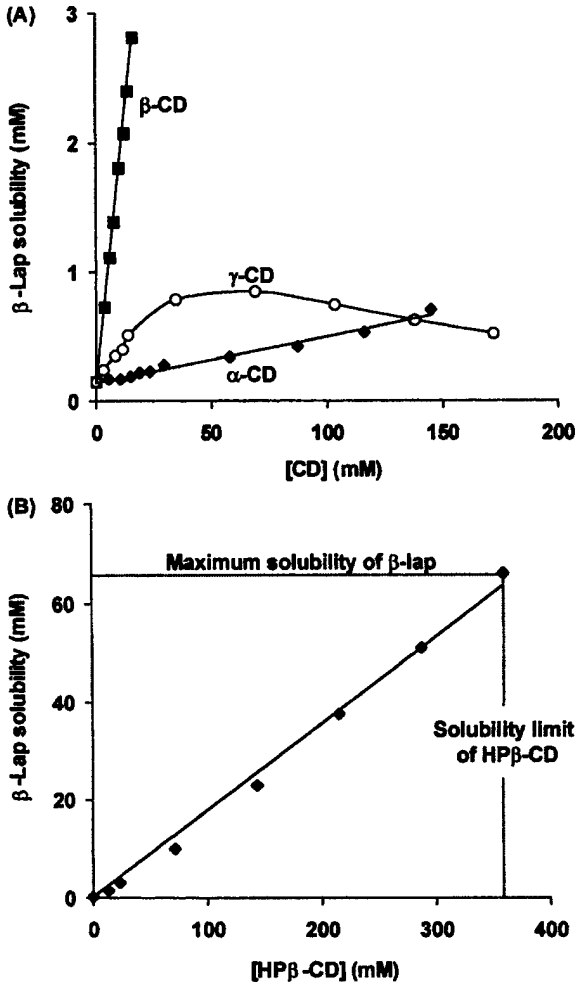

Fig. 2. Phase solubility diagrams of β-lap as a function of cyclodextrin concentrations at 25°C. A, α-CD, β-CD, and γ-CD. B, HPβ-CD.

These phase diagrams are classified as type A_L by Higuchi (14), which denotes a linear increase in solubility. In contrast, γ-CD showed a typical B_S-type solubility curve (14), which denotes an initial rise in the solubility of the solute followed by a plateau and a decreasing region because of the limited solubility of the complexes.

Increases in β-lap solubility in aqueous CD solutions are consistent with the formation of inclusion complexes between β-lap and CD molecules. In general, the main driving force for the complex formation is the hydrophobic interactions between a poorly soluble guest compound, such as β-lap, and the apolar cavity of the CD molecule. The hydrophobicity and geometry of the guest molecule as well as the cavity size of the CD molecule are important parameters for the complex formation. In the current study, the enhancement of β-lap solubility is highly dependent on the type of CD molecule. For example, the phase diagram for β-CD shows a much higher slope (0.16) than that of α-CD (0.0035) and the linear region ([γ-CD] < 20 mM) of γ-CD (0.024, Fig. 2A), demonstrating that β-CD is more effective in solubilizing β-lap. Based on the phase solubility diagrams, the association constants for the different inclusion complexes are determined using Eq. (1). The values of K_c are 20.0 ± 0.7, (1.23 ±

0.01) × 10^3, (0.94 ± 0.08) × 10^3, and 160 ± 5 M^{-1} for α–CD, β–CD, HPβ-CD, and γ-CD, respectively.

The different association constants for different cyclodextrin molecules indicate the importance of cavity size to encapsulate the β-lap molecule. α-CD has the lowest affinity to associate with β-lap, presumably because β-lap cannot fit into the relatively small hydrophobic cavity of α-CD (diameter ~5 Å, Table I). This is in agreement with other studies (10) in which guest molecules carried a phenyl moiety. On the other hand, although the wider cavity size of γ-CD (diameter ~8 Å) allows room for encapsulation (K_c increased by a factor of 8 for γ-CD over α-CD), it has lower affinity to associate with β-lap than that of β-CD and HPβ-CD, which have smaller cavity size. Therefore, β-CD and HPβ-CD appear to be significantly better host molecules for β-lap encapsulation. The much higher association constants of HPβ-CD and β-CD show the importance of appropriate cavity size in facilitating the interactions between β-lap and HPβ-CD or β-CD, as further supported by molecular recognition studies of host–guest chemistry (18).

Even though β-CD is a better host molecule for β-lap than α-CD and γ-CD, its application to maximize the solubility of β-lap is limited by the solubility of β-CD vehicle itself (16.3 mM). Consequently, the maximal solubility of β-lap in β-CD solution is limited to 2.8 mM or 0.68 mg/ml. This concentration is still relatively low for systemic administrations of this drug. To overcome this problem, we used HPβ-CD molecule as a β-lap carrier. HPβ-CD is formed by covalent modification of the external hydroxyl groups on β-CD by hydroxylpropyl groups. The modification significantly increased the solubility limit of HPβ-CD (360 mM, a factor of 22 over β-CD). The maximal solubility of β-lap in HPβ-CD solution reached 66.0 mM or 16.0 mg/ml, a 24-fold increase over that in β-CD vehicle and a 413-fold increase over β-lap aqueous solubility (0.16 mM). HPβ-CD provides the most effective candidate to solubilize β-lap.

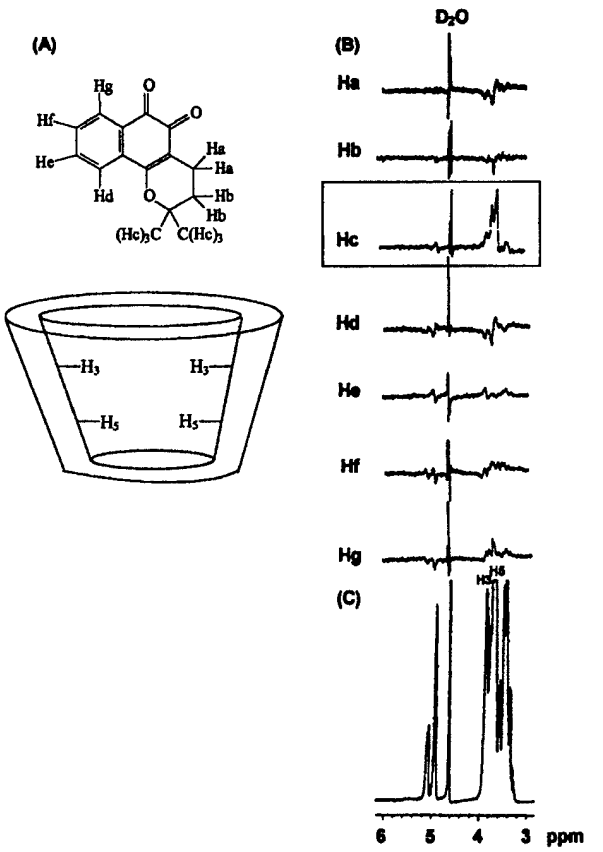

Fig. 3. A, Chemical structure of β-lap and general geometry of HPβ-CD. B, GROESY spectra of HPβ-CD·β-lap inclusion complex in D_2O at 25°C. C, The ^{1}H-NMR spectrum of HPβ-CD·β-lap inclusion complex. The concentrations of HPβ-CD and β-lap are 58.8 and 10.6 mM, respectively.

NMR Study of CD·β-Lap Inclusion Complexes

NMR spectroscopy is a powerful tool to study the inclusion phenomena. It has been shown that GROESY spectroscopy can be used to accurately detect the nuclear Overhauser effect (NOE) (19,20). In this study, we carried out the GROESY experiment to gain insight regarding the molecular structure of HPβ-CD·β-lap inclusion complex. Fig. 3 shows the GROESY spectra of the HPβ-CD·β-lap inclusion complex obtained by exciting every proton of β-lap (Ha to Hg). The significant NOE enhancement of the H5 and H3 protons located inside the HPβ-CD cavity was observed with the selective excitation of the Hc protons from β-lap. In contrast, no obvious NOE enhancement was observed with the selective excitation of the rest of β-lap protons, suggesting that the methyl moiety of β-lap is bound inside the cavity. This result also suggests that HPβ-CD forms a 1:1 inclusion complex with β-lap.

It is well known that the insertion of a guest molecule into the hydrophobic cavity of cyclodextrin can effect chemical shifts of the guest protons. In this experiment, we studied the effect of β-CD and HPβ-CD on the resonance of β-lap protons. Fig. 4A A shows the effect of increasing β-CD concentration on the ^{1}H-NMR spectra of phenyl protons of β-lap. Interestingly, Hd was the only proton that showed upfield shifts as a result of increasing β-CD concentrations. Above [β-CD] = 11.4 mM, no further changes of the upfield shift were observed (data not shown). Upfield shifts of Hd as a result of increasing of HPβ-CD concentrations were also found. Figure 4B shows the effect of β-CD on the ^{1}H-NMR spectra of methyl (Hc) and methylene (Ha, Hb) protons of β-lap. A splitting of these three groups of proton peaks was observed to result from the formation of an inclusion complex. This effect was most pronounced with the methyl protons (Hc), whereas the Ha protons had the least effect, suggesting the formation of diastereomeric complexes between β-lap and CD. A splitting of Ha, Hb, and Hc was also found with HPβ-CD, but the signal was interfered with by the methylene protons from hydroxypropyl groups on HPβ-CD.

The upfield shift of Hd (but not of other phenyl protons) and the splittings of Ha, Hb, and Hc indicate that these changes are the result of inclusion complex formation but not of the nonspecific interaction between cyclodextrin and β-lap. Chemical shift changes of Hd (Fig. 5A) A as a function of β-CD and HPβ-CD concentrations and the splitting of Hc (Fig. 5B) as a function of β-CD gave good fits with a 1:1 complex model as shown in Eq. (2) (15). The association constants determined from these data are 774 ± 52 M^{-1} (Hd shift) and 734 ± 20 M^{-1} (Hc splitting) for the β-CD·β-lap inclusion complex, and 662 ± 27 M^{-1} (Hd shift) for the HPβ-CD·β-lap inclusion complex.

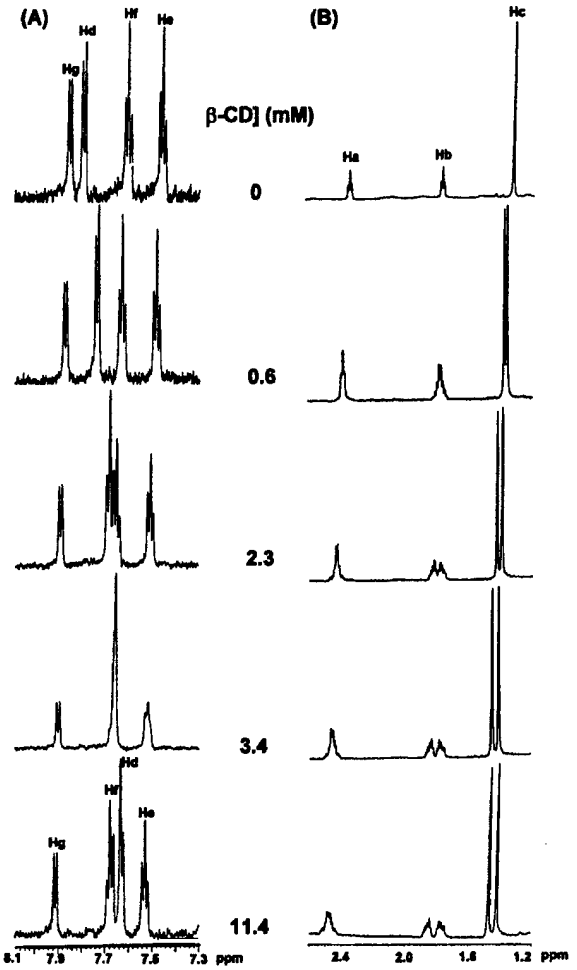

Fig. 4. ^{1}H-NMR (600 MHz) spectra of β-lap ([β-lap] = 0.123 mM) as a function of β-CD concentrations in D_2O. A, Phenyl protons (Hd, He, Hf, and Hg). B, Methyl and methylene protons (Ha, Hb, and Hc).

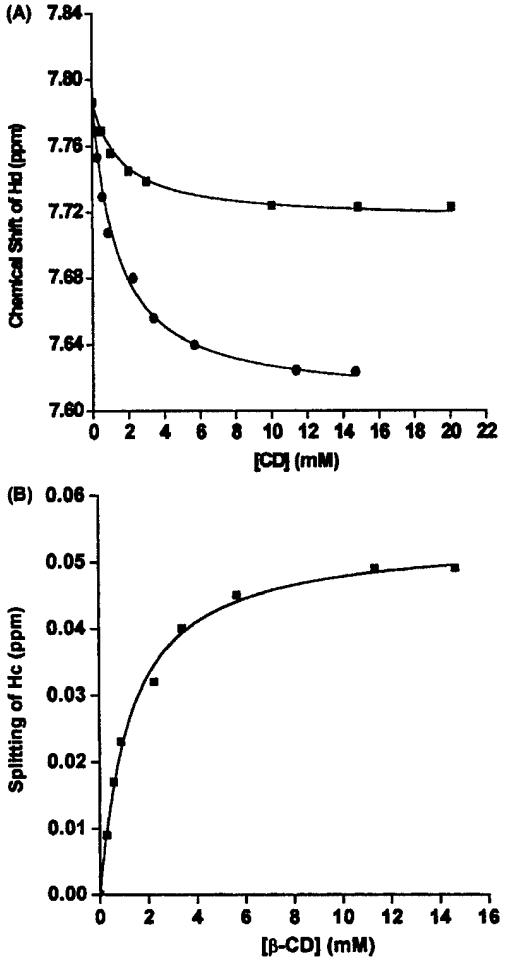

Fig. 5. Nonlinear curve fitting of Eq. (2) to experimental data using (A) chemical shift of Hd in β-lap ([β-lap] = 0.123 mM) as a function of HPβ-CD (■) and β-CD (●) concentrations in D_2O. B, Splitting of Hc as a function of β-CD concentrations in D_2O.

Fluorescence Studies of β-Lap Inclusion Complex

In the course of this study, we discovered that β-lap was a fluorescent molecule, and we used fluorescence spectroscopy to further study the association of HPβ-CD·β-lap and β-CD·β-lap inclusion complexes. Fig. 6A A shows a series of emission spectra of β-lap alone in PBS buffer at different excitation wavelengths ranging from 257 to 360 nm. These data showed that an excitation wavelength at 330 nm gave the highest emission intensity. For all the excitation wavelengths, the maximum emission wavelength was located at 436 nm. These experiments established the optimal spectroscopy conditions for β-lap complexation studies (λ_{ex} = 330 nm, λ_{em} = 436 nm).

Figure 6B shows the dependence of β-lap emission spectra as a function of HPβ-CD concentrations in PBS buffer. All the experiments were carried out at the same excitation wavelength (λ_{ex} = 330 nm) and same β-lap concentration (18 μM). Results showed that the β-lap emission intensity decreased when the HPβ-CD concentration increased (Fig. 6B). In addition, there is a slight blue shift (~6 nm) of the maximum emission wavelength in solution containing HPβ-CD. The change in fluorescence intensity and maximum emission wavelength of guest β-lap compound by addition of cyclodextrins is another indication of the formation of inclusion complexes between these two compounds. On encapsulation inside the hydrophobic cavity of CD molecules, the β-lap compound encounters a different chemical environment compared to aqueous solution. Geometric restrictions caused by space limitations in the CD cavity and reduced polarity because of the hydrophobic cavity of CD are found to alter the energetics and dynamics of the photophysical and photochemical processes of the guest molecule (21). The blue shift is consistent with the fact that β-lap experiences a less-polar environment in the hydrophobic cavity of HPβ-CD.

Next, we determined the association constant for the formation of the inclusion complex based on the fluorescence data. Emission intensity at 436 nm was used for these studies. Scatchard analysis by Eq. (3) (22) was used to determine the association constant (K_c) of the inclusion complex.

$$R/[CD]_f = n K_c - R K_c \quad (3)$$

where $[CD]_f$ is the unbound (free) molar concentration of CD, n is the number of binding sites, i.e., the stoichiometry

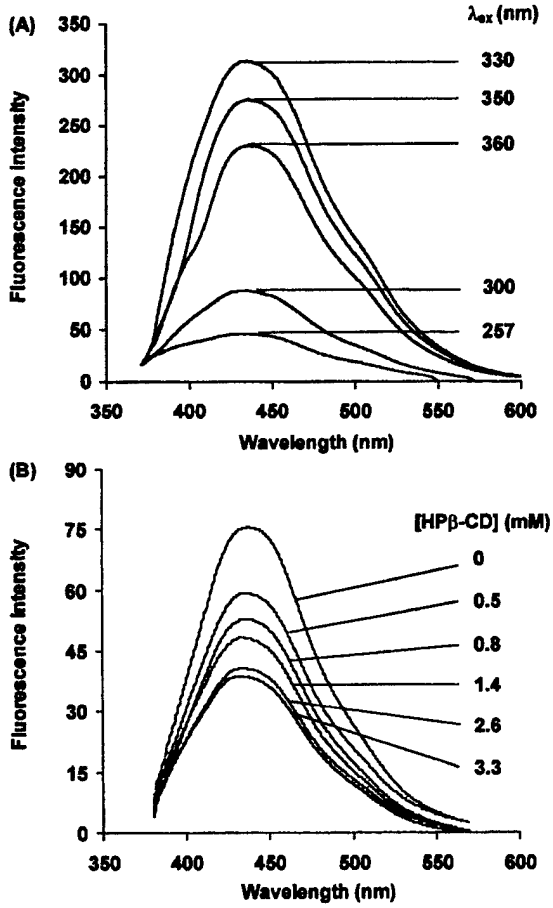

Fig. 6. A, Emission spectra of β-lap (61 μM) at different excitation wavelengths ranging from 257 to 360 nm. B, Emission spectra of β-lap (18 μM) in different HPβ-CD concentrations at 25°C (λ_{ex} = 330 nm).

of the complex, and R is the molar fraction of β-lap bound to CD.

The values of K_c are (1.10 ± 0.06) × 10^3 M^{-1} (R^2 = 0.97) and (1.06 ± 0.06) × 10^3 M^{-1} (R^2 = 0.98) for β-CD·β-lap and HPβ-CD·β-lap complexes, respectively. The numbers of binding sites (n) of β-CD·β-lap and HPβ-CD·β-lap inclusion complexes were found to be 1.04 ± 0.02 and 1.01 ± 0.02, respectively, which confirm the formation of 1:1 inclusion complexes. The values of K_c from fluorescence measurement are consistent with those from phase solubility studies but are higher than the data from NMR measurement. This difference is most likely a result of the different solvents used (e.g., PBS buffer was used in fluorescence and phase solubility studies, in comparison to D_2O in NMR studies).

In Vitro Cytotoxicity Studies in MCF-7 Cells

In order to evaluate the biologic activity of β-lap when it forms inclusion complexes with cyclodextrin, initial cytotoxicity DNA assays using MCF-7 human breast cancer cells were performed. Previous studies (13,16,23) have demonstrated that NQO1-expressing MCF-7 cells treated under these conditions not only showed growth inhibition, but the results can be equated to loss of survival according to colony-forming ability assays. Log-phase MCF-7 cells were exposed to different concentrations of β-lap in HPβ-CD inclusion complexes, β-lap in β-CD inclusion complexes, or with HPβ-CD and β-CD alone for 4 h. Drugs were then removed, and DNA content as a measure of cell survival was determined. β-Lap in DMSO was used as a positive control for comparison. Fig. 7 shows the viability of MCF-7 cells exposed to HPβ-CD·β-lap, β-CD·β-lap inclusion complexes, or with HPβ-CD and β-CD alone. The primary x-axis is the β-lap concentration used in this experiment, and the secondary x-axis is the concentration of HPβ-CD and β-CD required to solubilize β-lap. Cell viability of MCF-7 cells was statistically identical for cells treated with vehicles (HPβ-CD, β-CD) alone or with PBS for 4 h. These data showed that pure HPβ-CD (0 to 18.8 μM) and β-CD (0 to 20.8 μM) alone showed no cytotoxicity or growth inhibition. β-Lap in HPβ-CD and β-lap in β-CD inclusion complexes showed similar cytotoxic responses for the entire range of β-lap–equivalent doses (Fig. 7). Quantitatively, the drug potency was measured as TD_{50}, the toxic dose that kills 50% of the cell population. The TD_{50} values of β-lap in HPβ-CD and β-CD inclusion complexes were found to be the same at 2.1 μM for a 4-h transient drug exposure. These values were slightly higher than that from β-lap in DMSO, whose TD_{50} value is 1.7 μM.

In Vivo Analyses of β-Lap Toxicity

To evaluate the bioavailability of β-lap in CD inclusion complexes, C57Blk/6 mice were injected with increasing concentrations of β-lap in HPβ-CD inclusion complex 3 days per week for 3 weeks, and changes in weight and survival were recorded. Results showed no morbidity (decreases in weight loss) or lethality of mice for the control group injected i.p. with vehicles alone, or for mice injected i.p with 20 to 50 mg/kg of β-lap in HPβ-CD inclusion complex. In contrast, mice injected i.p. with 70 to 100 mg/kg showed both morbidity and 100% lethality (Fig. 8). Finally, mice treated with 60 mg/kg β-lap in HPβ-CD inclusion complex i.p. showed significant morbidity (loss of >15% body weight in most animals) and

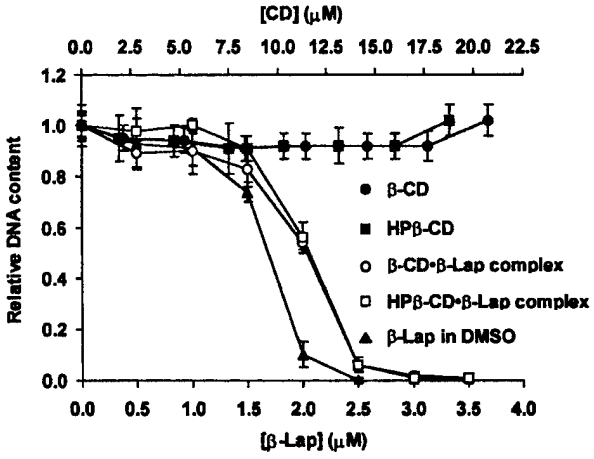

Fig. 7. Viability of log-phase MCF-7 cells exposed to β-lap in DMSO, HPβ-CD or β-CD inclusion complexes, as well as β-CD and HPβ-CD vehicles alone. For β-lap in DMSO and β-lap inclusion complexes, the bottom horizontal axis denotes the β-lap concentrations. The top horizontal axis denotes the cyclodextrin concentrations in β-lap inclusion complexes as well as for vehicles (β-CD and HPβ-CD) alone. Experiments were performed at least two times in triplicate to provide the standard deviation.

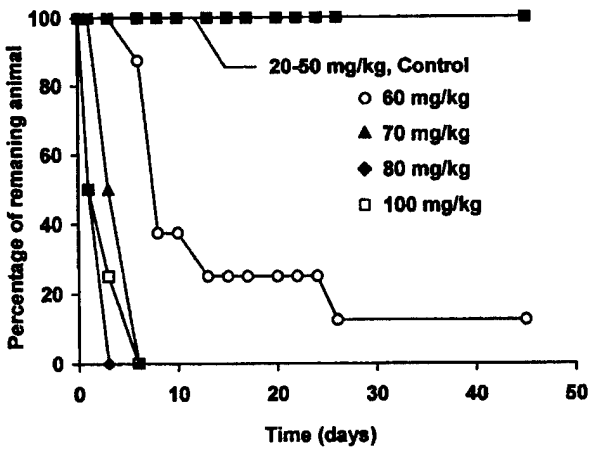

Fig. 8. Effect of varied doses of β-lap in HPβ-CD inclusion complex and HPβ-CD (control group injected with 5000 mg of HPβ-CD/kg) on the survival of C57Blk/6 mice. Animals were injected i.p. on days 1, 3, 6, 8, 10, 13, 15, 17, 20, and 22.

lethality (seven of eight animals died within 45 days of the treatment regimen). Consequently, the LD_{50} (lethal dose that kills 50% of mice population) value of β-lap in HPβ-CD inclusion complex was estimated to be 50–60 mg/kg in 18- to 20-g C57Blk/6 mice. This was determined by considering that 50 mg/kg kills 0% of mice, and 60 mg/kg kills 85% of mice in the course of this experiment. Interestingly, mice responded to doses above 50 mg/kg β-lap in HPβ-CD inclusion complex, but not to HPβ-CD vehicle alone, with unusual but temporary drug reactions. Within 15 min post-i.p.-injection, mice were observed to have a shivering reflex and difficulty in breathing. These drug responses lasted approximately 2 h, with mice exposed to 40–50 mg/kg recovering completely with essentially no weight loss noted over time. In contrast, most mice exposed to >60 mg/kg exhibited similar drug responses that resulted in lethality. Preliminary autopsies with mice that ultimately died did not result in the detection of major damage to vital organs, and more detailed analyses of cause of death are ongoing. Our studies indicate a nearly threefold greater bioavailability of β-lap *in vivo* compared to previous animal studies using Cremophor as a vehicle for β-lap administration, where an LD_{50} of >150 mg/kg was reported (24).

CONCLUSION

Phase solubility studies of β-lap in complexation with α-CD, β-CD, HPβ-CD, or γ-CD were carried out to overcome the problems of β-lap solubility and bioavailability. HPβ-CD demonstrated the maximum enhancement of β-lap solubility to 16.0 mg/ml or 66.0 mM, more than a 400-fold increase over β-lap solubility in water (0.038 mg/ml or 0.16 mM). The association constants of β-lap with cyclodextrins were determined by the phase solubility method, ^{1}H-NMR, and fluorescence spectroscopy (λ_{ex} = 330 nm, λ_{em} = 436 nm). β-CD and HPβ-CD showed higher binding affinity (K_c = 0.9–1.2 × 10^3 M^{-1}) to β-lap than α-CD (20 M^{-1}) and γ-CD (160 M^{-1}). Cytotoxicity assays indicated little differences in biologic activity between β-lap in HPβ-CD or β-CD inclusion complexes, with nearly identical cell responses (cell death in induced apoptosis) and TD_{50} values (2.1 μM). Finally, studies of morbidity and mortality in C57Blk/6 mice suggested a LD_{50} of 50–60 mg/kg, with no morbidity or mortality following 20–50 mg/kg β-lap in HPβ-CD inclusion complex. Complexation of β-lap with HPβ-CD offers a major advancement in improvement of bioavailability of this very active anticancer agent, and the antitumor activity of these complexes against human breast and prostate cancer xenografts are under investigation.

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Development of β-lapachone prodrugs for therapy against human cancer cells with elevated NQO1 levels*

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Running Title: β-Lapachone derivatives as potential prodrugs

Key Words: β-Lapachone, β-Lapachone derivatives, Apoptosis, Breast cancer, Prodrugs, NQO1 (DT-diaphorase)

[9]**Abbreviations used were:** β-Lap, β-Lapachone; 231-NQ6, $NQO1^+$ MDA-MB-231 clone #6; 231-NQ2, $NQO1^-$ MDA-MB-231 clone #2; 468-NQ3, $NQO1^+$ MDA-MB-468 clone #3; 468-Vec4, $NQO1^-$ MDA-MB-468 vector alone clone #4; BPIL, *p*-bromophenylimine lapachone; BSA, bovine serum albumin; DEVD, Acetyl-Asp-Glu-Val-Asp-aldehyde; Dic, Dicoumarol; HP-β-CD, hydroxypropyl-β-cyclodextrin; IR, ionizing radiation; LD_{50}, dose in μM that caused 50% lethality; MCF-7, $NQO1^+$ MCF-7:WS8; Men, menadione; MePIL, *p*-methylphenylimine lapachone; MPIL, *p*-methoxyphenylimine lapachone; NAC, *N*-acetyl-L-cysteine; NPIL, *p*-nitrophenylimine lapachone; NQO1, NAD(P)H:quinone oxidoreductase 1 (DT-diaphorase or x-ray-inducible gene/transcript #3 (E.C. 1.6.99.2)); PARP, poly(ADP-ribose) polymerase; PIL, phenylimine lapachone; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; zVAD, N-benzyloxycarbonyl-Tyr-Val-Ala-Asp-aldehyde.

SUMMARY

β-Lapachone, an *o*-naphthoquinone, induces a novel caspase- and p53-independent apoptotic pathway dependent on NAD(P)H:quinone oxidoreductase 1 (NQO1) expression. NQO1 reduces β-lapachone to an unstable hydroquinone that rapidly undergoes a two-step oxidation back to the parent compound, perpetuating a futile death cycle (1). A deficiency in NQO1, or inhibiting NQO1 with dicoumarol rendered cells resistant to β-lapachone cytotoxicity. Thus, β-lapachone has great potential for the treatment of specific cancers with elevated NQO1 levels (e.g., cancers of the breast, lung, pancreas, colon, and prostate) compared to adjacent normal tissue.

We report the development of mono(arylimino) derivatives of β-lapachone (2) as potential prodrugs. These derivatives were relatively non-toxic and not substrates for NQO1 when initially solubilized in water. In solution, however, they underwent hydrolytic conversion to β-lapachone at rates dependent on the electron-withdrawing strength of their substituent groups. NQO1 enzyme assays, UV-vis spectrophotometry, HPLC-ESI-MS, and NMR analyses were used to monitor and confirm the conversion of each derivative to β-lapachone.

Once converted, β-lapachone derivatives caused NQO1-dependent, μ-calpain-mediated cell death responses in human breast cancer cells similar to those caused by β-lapachone. Interestingly, co-administration of *N*-acetyl-L-cysteine, an efficient reactive oxygen scavenger, prevented derivative-induced cytotoxicity, but did not affect β-lapachone lethality. However, *N*-acetyl-L-cysteine inactivation of β-lapachone derivative cytotoxicities was the result of direct reactive modification of these derivatives, that prevented their conversion to β-lapachone. The use of β-lapachone mono(arylimino)

prodrug derivatives, or more specifically a derivative converted in a tumor-specific manner (i.e., in the acidic local environment of the tumor tissue), could reduce normal tissue toxicity while causing tumor-selective cell killing through NQO1.

INTRODUCTION

β-Lapachone (β-Lap[9], 3,4-dihydro-2,2-dimethyl-2*H*-naphtho[1,2-*b*]pyran-5,6-dione) has significant antitumor activity against sarcoma 180 (S-180) cells *in vitro*, and in mice bearing S-180 ascites tumors (3). Further studies showed that β-lap elicited significant antitumor activity against Yoshida sarcoma and Walker 256 carcinoma cells (3,4). Recent data showing activity against a variety of tumors has revitalized interest in this drug (3-6).

Recent studies revealed that β-lap was "bioactivated" by the two-electron oxidoreductase, NAD(P)H:quinone oxidoreductase 1 (NQO1, E.C. 1.6.99.2,(1)). Structural similarities between β-lap and other members of the naphthoquinone family, such as menadione (vitamin K_3, 2-methyl-1,4-naphthoquinone), suggested that NQO1 may be involved in the activation or detoxification of this drug (7-11). Furthermore, the ionizing radiation (IR)-inducible properties of NQO1 (i.e., xip3) were consistent with this compound's ability to radiosensitize various human cancer cells (12). We subsequently discovered that NQO1 was a major intracellular determinant of β-lap cytotoxicity (1,13), and noted that all $NQO1^+$ cell lines examined were sensitive to β-lap. Furthermore, the cytotoxicity of β-lap was reversed by co-administration of dicoumarol (an NQO1 inhibitor), and cells lacking NQO1 were inherently resistant. In contrast, NQO1-overexpressing cancer cells were resistant to menadione, and co-administration of dicoumarol potentiated menadione lethality.

Previously, we showed that β-lap killed cells through a unique apoptotic pathway. Apoptosis is a form of cell death caused by many chemotherapeutic agents, and typically involves activation of cysteine protease zymogens (e.g., caspases (14,15)).

Apoptosis induced by β-lap in $NQO1^+$ human breast or prostate cancer cell lines was unique, causing a pattern of poly(ADP-ribose) polymerase (PARP) and p53 proteolysis *in vivo* that was distinct from events caused by agents (e.g., staurosporine) that stimulated cell death through caspase effectors (1,13,16-18). We previously noted concomitant atypical PARP (yielding an ~60 kDa fragment) and p53 cleavage (yielding an ~40 kDa fragment) events in β-lap-treated, $NQO1^+$ cells that were caused by the activation of the neutral, Ca^{2+}-dependent cysteine protease, μ-calpain (18-20). Further analyses showed that while certain aspects of β-lap cytotoxicity were unique (e.g., atypical PARP and p53 proteolysis), other aspects conformed to classic apoptosis (e.g., lamin B proteolysis, DNA condensation, TUNEL+ cells, sub-G_0/G_1 cells, trypan blue exclusion (13)).

Although β-lap has properties ideally suited for use as an antitumor agent, by targeting an enzyme typically elevated in various tumors, concerns over normal tissue toxicity remain. These concerns could be avoided by the development of a β-lap prodrug that would favor conversion to β-lap in the micro-environment of the tumor, and less so in normal tissues. β-Lap holds great promise as an antitumor agent because it: (a) is 'bioactivated' by NQO1 through a futile redox cycle, and NQO1 levels are elevated in numerous cancers; (b) kills independent of cell cycle, caspase, pRb or p53 status; and (c) synergistically kills cells in combination with chemotherapeutic agents, as well as IR (21). Ideally, prodrugs of β-lap would become active in a tumor-selective or spontaneous manner with identical or more potent antitumor activity as β-lap. The active form of such prodrugs would kill in the same NQO1-dependent manner, but avoid normal tissue toxicity depending on their mechanism and rate of activation.

In this study, we report that inactive mono(arylimino) derivatives of β-lap are converted to β-lap through a spontaneous Schiff's base hydrolytic reaction. The rates of hydrolyses of β-lap derivatives varied as a function of the strength of the electron-withdrawing substituent groups in the para position of the mono(arylimino) leaving group (Figure 1, R group). Our findings suggest that mono(arylimino) derivatives may be utilized as nontoxic, inactive, pH-sensitive precursors to β-lap. These derivatives may be advantageous for future delivery strategies *in vivo* for anti-cancer chemotherapy, causing limited normal tissue damage and therefore less severe side-effects, that are often associated with currently used chemotherapies.

EXPERIMENTAL PROCEDURES

Reagents—β-Lap (3,4-dihydro-2,2-dimethyl-2*H*-naphtho[1,2-*b*]pyran-5,6-dione) was synthesized by us, confirmed by NMR analyses, dissolved in DMSO at 40 mM and concentrations verified by spectrophotometric analyses (13,16). β-Lap stock solutions were stored at –80 °C until used. β-Lap mono(arylimino) derivatives (PIL, phenylimine, 2,2-dimethyl-(*Z*)-6-phenylimino-3,4,5,6-tetrahydro-2*H*-naphtho[1,2-*b*]oxin-5-one; MePIL, *p*-methylphenylimine, 2,2-dimethyl-(*Z*)-6-(4-methyl-phenylimino)-3,4,5,6-tetrahydro-2*H*-naphtho[1,2-*b*]oxin-5-one; MPIL, *p*-methoxyphenylimine, 2,2-dimethyl-(*Z*)-6-(4-methoxyphenylimino)-3,4,5,6-tetrahydro-2*H*-naphtho[1,2-*b*]oxin-5-one; NPIL, *p*-nitrophenylimine, 2,2-dimethyl-(*Z*)-6-(4-nitrophenylimino)-3,4,5,6-tetrahydro-2*H*-naphtho[1,2-*b*]oxin-5-one were synthesized as described (2). BPIL, *p*-bromophenylimine, 2,2-dimethyl-(*Z*)-6-(4-bromophenylimino)-3,4,5,6-tetrahydro-2*H*-naphtho[1,2-*b*]oxin-5-one) was prepared from 4-bromoaniline and β-lapachone as described previously (2) with 90% yield as a brown solid that was recrystalized from cyclohexane. ^{1}H NMR ($CDCl_3$, 200 MHz): d 8.19 (dd, *J* = 7.1 and 1.7, 1H, H-7), 7.83 (dd, *J* = 7.1 and 1.7, 1H, H-10), 7.50 (m, 2H, H-8, 9), 7.43 (d, *J* = 8.8, 2H, H-2′), 6.62 (d, *J* = 8.8, 2H, H-3′), 2.41 (t, *J* = 6.7, 2 H, H-4), 1.79 (t, *J* = 6.7, 2 H, H-3), 1.44 (s, 6 H, 2-CH_3); ^{13}C NMR ($CDCl_3$, 50 MHz): d 177.5 (C-5), 161.6 (C-10b), 152.1 (C-1′), 150.9 (C-6), 132.4 (C-10a), 131.7 (C-3′), 131.3 (C-8), 130.2 (C-9), 130.0 (C-6a), 127.0 (C-7), 123.3 (C-10), 117.6 (C-2′), 115.5 (C-4′), 111.9 (C-4a), 78.5 (C-2), 31.7 (C-3), 26.7 (2-CH_3), 16.0 (C-4). EIMS, m/z (%) 397 (M^+ + 2, 43), 395 (M^+, 43), 316 (48), 260 (93), 232 (100), 203 (35), 76 (32), 41 (37). Aryliminoquinones were dissolved in DMSO at 50 mM. Menadione was obtained from Sigma Chemical Co. (St. Louis, MO), dissolved in DMSO

at 100 mM, and stored at –80 °C. Drugs (β-lap, menadione, and β-lap mono(arylimino) derivatives) were added to media at a 1:1000 dilution immediately before administration to cells; control cells received ≤0.1% DMSO for vehicle alone treatment. Dicoumarol (Sigma) was used as described (1). NAC (Sigma) was dissolved in water, the pH adjusted to 7.4 using NaOH, and stored at 4 °C. Acetonitrile (HPLC grade), methanol (HPLC grade) and DMSO (ACS Certified Spectranalyzed grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Ammonium formate was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Medipure grade liquid nitrogen was obtained in high pressure, gas-withdrawal containers from Praxair, Inc. (Cleveland, OH, USA) for use as the sheath and auxiliary gas in the electrospray ion source of the mass spectrometer. Argon (5.0 Ultrahigh purity grade) was obtained from Praxair for use as the collision gas during tandem mass spectrometric experiments.

Cell Culture—NQO1-deficient ($NQO1^-$) MDA-MB-468 and MDA-MB-231 cells were obtained from the American Type Culture Collection and stably transfected with the CMV-driven human NQO1 cDNA in the pcDNA3 mammalian expression vector as described (1). $NQO1^+$ 468-NQ3 and 231-NQ6 cell lines, as well as $NQO1^-$ vector alone 468-Vec4 and 231-NQ2 were isolated. Clone 231-NQ2 was transfected with CMV-NQO1, exhibited neomycin resistance, but did not express NQO1 protein or enzymatic activity. MCF-7:WS8 (MCF-7) cells were obtained and grown as described (1). Tissue culture components were purchased from Life Technologies, Inc., unless otherwise stated. All cells were grown in RPMI 1640 media supplemented with 200 μg/ml Geneticin (except for parental MCF-7 cells), 5% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin (13,16). Cells were routinely

passed at 1:5 dilutions once per week using 0.1% trypsin-EDTA (1). All cells were grown in a 37 °C humidified incubator with a 5% CO_2, 95% air atmosphere (13,16) and routinely monitored for, and were free of, mycoplasma contamination (1).

Cell Growth Assays—Cell growth inhibition was assessed using DNA content assays as described (1,13). Briefly, MCF-7, 231-NQ6, 231-NQ2 (seeded at 1 x 10^4 cells/well), 468-Vec4 or 468-NQ3 cells were seeded (at 4 x 10^4 cells/well) in 48-well plates and allowed to attach overnight. Cells were then treated with 4 h pulses of varying drug concentrations (0-20 μM of β-lap, menadione, or each β-lap mono(arylimino) derivative, Figure 2) alone or in conjunction with 40 μM dicoumarol (dic) or 5.0 mM NAC; 40 μM dicoumarol was previously shown to prevent NQO1 activities in all cells examined, and 5.0 mM NAC was routinely used at this dose to scavenge reactive oxygen and nitrogen species (22). Drug-containing media were then removed, fresh media added, and cells allowed to grow for at least 7 days. DNA content (a measure of cell growth and survival) was determined by fluorescence using a Perkin Elmer HTS 7000 Bio Assay Reader microtitre plate reader (1). Data were expressed as relative growth, T/C (treated/control), from three wells per treatment. Each experiment was repeated at least three times, and data expressed as mean ± S.E.M. Prior analyses with β-lap and other β-lap analogs demonstrated that cytotoxicity monitored by growth assays correlated directly with changes monitored by colony forming ability assays (1).

UV-vis Spectrophotometric Analysis—UV-vis spectrophotometric analyses of the hydrolysis of β-lap mono(arylimino) derivatives were performed in the presence or absence of 5 mM NAC, using a Beckman DU 640 spectrophotometer

(Beckman Coulter, Fullerton, CA) scanning 200-500 nm wavelengths. Stock solutions (50 mM) of each β-lap mono(arylimino) derivative dissolved in DMSO were diluted to 1 ml with water and subjected to UV-vis wavelength scans every 30 min for 4 h. Scans were compared to similar analyses of β-lap dissolved in an identical manner.

NQO1 Enzyme Assays—S9 supernatants were prepared as described (1). Briefly, cellular extracts were prepared and resuspended in a small volume of PBS, pH 7.2, containing 10 μg/μl aprotinin. Cell suspensions were sonicated on ice four times using 10 sec pulses, and centrifuged at 14,000 x *g* for 20 min. S9 supernatants were aliquoted and stored at –80 °C until use. NQO1 enzyme activities were measured as described (1). Reaction medium contained 77 μM cytochrome c (practical grade; Sigma) and 0.14% bovine serum albumin in Tris-HCl buffer (50 mM, pH 7.5). Reactions were performed at 37 °C and initiated by the addition of S9 supernatants. NQO1 activity was measured using NADH (200 μM) as the immediate electron donor and β-lap, menadione, or β-lap mono(arylimino) derivatives (10 μM) as intermediate electron acceptors using a Beckman DU 640 spectrophotometer (Beckman Coulter, Fullerton, CA). Each assay was repeated in the presence of 10 μM dicoumarol, and activity attributed to NQO1 was that level inhibited by dicoumarol (23). Enzyme activities were calculated as nmol of cytochrome c reduced/min/μg protein, based on the initial rate of change in absorbance at 550 nm, and an extinction coefficient for cytochrome c of 21.1 mM/cm. Results shown are the average enzyme activity for three separate cell extracts ± S.D.

NADH Recycling Assays—NADH recycling assays were performed as described (1) using NQO1-containing S9 extracts from MCF-7 cells (as described

above). S9 extracts were incubated with 200-500 μM NADH in 50 mM Tris-HCl, pH 7.5, containing 0.14% bovine serum albumin. Reactions were initiated by addition of 10 μM β-lap, menadione or mono(arylimino) derivatives unless otherwise indicated. NADH oxidation was monitored by changes in absorbance at 340 nm measured for 5 min with a Beckman DU 640 spectrophotometer (Beckman Coulter, Fullerton, CA). Protein concentrations were determined by Bradford assays (1). Enzyme activities were expressed as treated/control (T/C) where moles NADH reduced in 5 min/mole substrate (β-lap, menadione, or mono(arylimino) derivative) added per μg protein are compared to moles NADH reduced in 5 min/mole menadione added per μg protein.

Western Blot Analyses—Western blot analyses of DMSO control or drug-treated MCF-7 cells were performed as described (13,16,18). Briefly, whole cell extracts were prepared by direct lysis of scraped, PBS-washed cells (both floating and attached cells were pooled) in buffer (6 M urea, 2% SDS, 10% glycerol, 62.5 mM Tris-HCl, pH 6.8, 5% β-mercaptoethanol, and 5 μg/ml bromophenol blue) followed by sonication. Equal protein amounts were separated by 10% SDS-PAGE and proteins of interest were visualized by standard western blot techniques (1) via incubation with Super Signal (Pierce) followed by X-ray film exposure. Loading equivalence and transfer efficiency were monitored by Western blot analyses of proteins not altered in cells following β-lap exposure (13). Dilutions of 1:2,000 for α-PARP SC-8007 antibody (Santa Cruz, Santa Cruz, CA) and 1:2,000 for α-p53 D01 antibody (Santa Cruz, Santa Cruz, CA) were used to monitor apoptotic proteolyses of these proteins.

^{1}H NMR Spectroscopy—One milligram (1.0 mg) MPIL was dissolved in deuterated DMSO (DMSO-d^6), diluted into water, and incubated at room temperature

for ≥4 h. Samples were filtered (0.2 μm nylon syringe filter), freeze-dried in a Labconco Freezone 4.5 lyophilizer, and re-dissolved in 1 ml DMSO-d^6 before acquiring NMR spectra. All ^{1}H NMR spectra were recorded on a 200 MHz Varian Gemini Fourier transform NMR spectrometer.

High pressure liquid chromatography-electrospray ionization-mass spectrometry (HPLC-ESI-MS)—Chromatographic separations were performed using the reverse - phase medium Polaris C18-A (3 μm particle diameter) contained in a 0.46 cm. i.d. x 5.0 cm l. column obtained from Ansys Metachem (Lake Forest, CA U.S.A.). The chromatographic mobile phase consisted of aqueous ammonium formate (2.5×10^{-2} M, pH not adjusted):acetonitrile (30:70 v:v). The column temperature and mobile phase flow rate were 40 °C and 0.5 ml/min, respectively, during all chromatographic separations. The mass spectrometer was operated with heated capillary and analyzer manifold temperatures of 350 °C and 70 °C, respectively. Ion source sheath gas pressure was 80 p.s.i.g., and the auxiliary gas flow rate was set to 40 dimensionless units. The instrument was operated in positive ion mode, the ion source electrospray potential was 3.5 kV, and the collision cell argon pressure was 2.0×10^{-5} milliTorr. β-Lap and β-lap mono(arylimino) derivatives ionized readily as positively charged proton adducts and underwent a characteristic collision - induced loss of m/z 56 in the mass spectrometer. Transitions were monitored (precursor ion (m/z), product ion (m/z), collision cell offset potential (V)): β-lap (243, 187, -24), PIL (318, 262, -25), MePIL (332, 276, -25), MPIL (348, 292, -25), NPIL (363, 307, -25), and BPIL (396, 340, -25). Acquisition dwell time and product ion scan width were 0.1 sec and m/z 1.0, respectively, for all monitored transitions. Chromatographic peaks were observed for β-

lap, PIL, MePIL, MPIL, NPIL, and BPIL at 2.0, 5.1, 6.3, 4.6, 5.1, and 7.8 min, respectively.

Measurements of β-Lap Mono(arylimino) Derivative Hydrolysis—β-Lap mono(arylimino) derivative stock solutions in DMSO (~3 x 10^{-3} M) were diluted 10:1000 v:v in methanol. These methanolic working solutions were diluted immediately 10:1000 v:v in water contained by autosampler vials. Vials were capped with teflon-lined septa, and transferred to the automatic liquid sampler of the HPLC-ESI-MS system. Aliquots (5 µl) were injected into the instrument at precisely measured intervals of ~1 h for the 30 h duration of the experiment. β-Lap mono(arylimino) derivative concentration vs. time data sets were modeled as an irreversible first order process according to the equation:

$$C(t)=C0+a*\exp(-k*x)$$

The production of β-lap during β-lap mono(arylimino) derivative hydrolysis was modeled as an irreversible first order process according to the equation:

$$C(t)=C0+a*(1-\exp(-k*x))$$

RESULTS

Phenylimine lapachone (PIL), methylphenylimine lapachone (MePIL) and methoxyphenylimine lapachone (MPIL) β-lap mono(arylimino) derivatives mimic β-lap cytotoxicity—Various mono(arylimino) substituted β-lap derivatives were synthesized (2) wherein each mono(arylimino) leaving group was unique due to its electron-withdrawing strength (Figure 1). Since NQO1 reduces quinones (either in the para or ortho positions (2)), mono(arylimino) derivatives of β-lap were not expected to be substrates for NQO1, nor were they expected to selectively kill $NQO1^+$ human cancer cells as noted with β-lap (1). To determine the cytotoxicity of each β-lap mono(arylimino) derivative, we performed relative growth assays using $NQO1^+$ (e.g. 468-NQ3, 231-NQ6, and MCF-7 cells) compared to $NQO1^-$ (e.g. 468-Vec4, and 231-NQ2) cells. We previously demonstrated that relative growth assays directly correlate with lethality responses monitored by colony forming ability assays using $NQO1^+$ *versus* $NQO1^-$ breast cancer cells after β-lap exposure (1). Log-phase cells were exposed for 4 h to various concentrations of β-lap alone, menadione alone, each β-lap mono(arylimino) derivative alone, or each of these compounds in combination with 40 μM dicoumarol (Figure 2). β-Lap mono(arylimino) derivatives PIL, MePIL, and MPIL were as selectively toxic to $NQO1^+$ cells as β-lap, with comparable LD_{50} values of 5.9 ± 1.3 μM, 5.7 ± 1.0 μM, 3.7 ± 0.5 μM and 6.6 ± 1.4 μM, respectively (Figure 2, Table I). In contrast, NPIL and BPIL were significantly less toxic to $NQO1^+$ cells with LD_{50} values of >50 μM (Figure 2, Table I); these assays had an upper limit of 50 μM, indicating that NPIL and BPIL were relatively nontoxic. Dicoumarol prevented PIL, MePIL, and MPIL cytotoxicity in a manner similar to that observed with β-lap in $NQO1^+$ cells, shifting LD_{50}

values to 21 ± 0.5 μM, 30 ± 1.0 μM, 17.8 ± 1.9 μM and 26 ± 0.5 μM respectively. NQO1$^-$ 468 and 231 cells were resistant to all drugs examined. Overall, these data suggested that these β-lap mono(arylimino) derivatives may be converted to β-lap or β-lap-like compounds to kill in an NQO1-dependent manner.

Evidence for conversion of β-lap mono(arylimino) derivatives to β-lap—Direct data supporting the hypothesis that β-lap mono(arylimino) derivatives were converted to β-lap was then sought. Stock solutions of β-lap mono(arylimino) derivatives were dissolved in water and subjected to UV-vis spectrophotometric wavelength scans of 200-500 nm every 30 min for 4 h, representing the pulse used in cytotoxicity experiments (Figure 2). During that time, we observed the UV-vis spectra of cytotoxic β-lap mono(arylimino) derivatives significantly change, resembling that of β-lap (Figure 3*A-C*). In contrast, scans of β-lap remained unaltered over a 4 h period. These data confirmed that each of these β-lap mono(arylimino) derivatives were spontaneously converted to β-lap in 4 h (Figure 3*A-C*, right panels). In contrast, scans of NPIL and BPIL, less toxic derivatives, were initially (time 0), and after 4 h, different from scans of β-lap (Figure 3*D,E*), suggesting that these compounds had low or no conversion rates to β-lap.

Using HPLC-ESI-MS, we monitored the disappearance of PIL, MePIL, MPIL and NPIL, as well as accumulation of β-lap over time (Table II). The apparent ranked rates of conversion of these β-lap derivatives from highest to lowest were: MPIL>MePIL>PIL>NPIL, with rate constants of derivative loss of –0.693 to –0.001 h^{-1}, and rate constants of β-lap formation of 0.221 to 0.034 h^{-1}. NMR spectra before and after incubating MPIL with water ≥4 h confirmed the disappearance of the

mono(arylimino) derivative and an increase in β-lap formation (data not shown). The rates of loss of parent compounds (Table II) correlated well with concentrations required for cytotoxicity (Table I, and Figure 2). For example, the low rate of conversion of NPIL to β-lap was consistent with the lowered cytotoxicity of this set of compounds (e.g., NPIL, BPIL).

β-Lap mono(arylimino) derivatives become NQO1 substrates—Data from Figures 2, 3, and Table I suggest that β-lap mono(arylimino) derivatives were converted to β-lap. Therefore, we performed NQO1 enzyme activity assays after immediate (t = 0), 1 or 4 h of dissolving each β-lap derivative in aqueous buffer. NQO1 activities would, thereby, reflect conversion of β-lap mono(arylimino) derivatives to β-lap. For comparison, β-lap and menadione were included as controls. S9 supernatants from MCF-7 cells were used as a source of NQO1 for enzymatic reactions. NQO1 activity was measured using NADH as the immediate electron donor, and menadione as the intermediate electron acceptor as described in "Experimental Procedures." As expected, upon immediate addition of drug to the reaction buffer (t = 0), all β-lap mono(arylimino) derivatives examined were poor NQO1 substrates, reflected by low NQO1 activities (1.17-6.49 μmol cyt c/min/μg) compared to menadione (21.23 μmol cyt c/min/μg) or β-lap (13.34 μmol cyt c/min/μg, Figure 4*A* t = 0).

However, after a 1 h incubation in reaction buffer without cytochrome c, PIL, MePIL, and MPIL became NQO1 substrates, eliciting NQO1 enzyme activities ranging from 9.48-13.49 μmol cyt c/min/μg (Figure 4*A*, t = 1 h), similar to β-lap at time 0. In contrast, the relatively nontoxic derivatives, BPIL and NPIL, remained poor substrates for NQO1 (1.34-2.25 μmol cyt c/min/μg). As expected after an incubation time of 4 h,

PIL, MePIL, and MPIL became better substrates for NQO1, eliciting approximately three to five times the level of NQO1 enzyme activity initially observed at t = 0, rising to 14.7-18.6 μmol cyt c/min/μg (Figure 4*A*, t = 4 h). NPIL elicited approximately twice the level of NQO1 enzyme activity (2.9 μmol cyt c/min/μg), and BPIL elicited approximately three times the level of NQO1 enzyme activity initially observed at time 0, rising to 4.6 μmol cyt c/min/μg. These data suggest that, while incubating in aqueous solution, these β-lap mono(arylimino) derivatives were converted into NQO1 substrates at rates that correlated with the cytotoxicities of each β-lap mono(arylimino) derivative in $NQO1^+$ cells (Figure 2) and the hydrolysis rates determined by HPLC-ESI-MS (Table II).

A unique feature of β-lap is that when NQO1 metabolizes this drug in the absence of an electron acceptor (e.g., cytochrome c), a futile cycle of NADH oxidation occurs (1). Up to 10 moles of NADH may be oxidized per mole of β-lap in 5 min (Figure 4*B*). In contrast, when menadione was used in the same reaction, approximately one mole of NADH was oxidized per mole menadione in 5 min, a known detoxification reaction in the cell whereby menadione is subsequently conjugated with glutathione and detoxified (24). Except for MPIL, when β-lap mono(arylimino) derivatives were examined immediately after solubilization into water, these compounds were not NQO1 substrates and therefore elicited minimal futile redox cycling in this enzymatic assay. Consistent with the notion that PIL, MePIL and MPIL were converted to β-lap, a known NQO1 substrate (1), at 4 h these same compounds elicited significant NQO1 futile cycling; for example, 7.25-12.9 fold treated/control (T/C) was noted, essentially equal to the futile cycling noted for β-lap (9.98 fold T/C). Consistent with the lowered cytotoxicity

of NPIL and BPIL (Figure 2, Table I), these compounds elicited much lower or no NQO1-dependent recycling activities (2.37-2.73 fold T/C, Figure 4*B*).

β-Lap mono(arylimino) derivatives induce atypical PARP and p53 cleavage in NQO1$^+$ MCF-7 cells—β-Lap-induced apoptosis is defined by atypical proteolyses of PARP (resulting in an ~60 kDa fragment from the 113 kDa full-length protein (1,16,18,25)) and p53 (giving an ~40 kDa fragment (18)), detected by Western blotting (Figures 5*A* and 6*A*). These proteolytic events correlate well with lethality caused by β-lap exposure, and are indicative of μ-calpain-mediated cell death responses (18). NQO1$^+$ MCF-7 cells were treated for 4 h with PIL, MePIL, and MPIL (separate exposures, each at 5 μM), or NPIL and BPIL (separate exposures, each at 20 μM); since NPIL and BPIL were relatively nontoxic at concentrations below 15 μM (Figure 2), we used higher concentrations of these compounds to examine apoptotic proteolyses. Consistent with their abilities to elicit cell death, PIL, MePIL, and MPIL induced atypical PARP (Figure 5*B-D*) and p53 proteolyses (Figure 6). These proteolytic events were abrogated with 40 μM dicoumarol (dic) co-treatment as shown for β-lap (1), and did not occur in NQO1$^-$ 231 or 468 cells (data not shown). Unlike β-lap, PARP and p53 proteolyses induced by PIL, MePIL, or MPIL were blocked by co-treatment with 5 mM NAC (Figures 5 and 6), consistent with the protection afforded by NAC against PIL, MePIL, and MPIL cytotoxicities (Table I). NPIL and BPIL did not induce atypical proteolyses of PARP or p53 (Figures 5 and 6), consistent with their inability to induce apoptosis or lethality after a 4 h exposure (Figure 2, Table I).

NAC prevents β-lap mono(arylimino) derivative conversion to β-lap and subsequent cytotoxicity—Co-administration of NAC did not affect the cytotoxicity of β-

lap, suggesting that reactive oxygen species (ROS) produced by this drug may not play a role in its lethality, or that the ROS formed by treatment of $NQO1^+$ cells with β-lap are compartmentalized (Table I); in fact, preliminary data indicate that NAC pre- and post-treatment of cells did not block ROS formation (Reinicke et al., unpublished data). In contrast, co-treatments of $NQO1^+$ cells with 5 mM NAC in combination with PIL, MePIL or MPIL dramatically protected these cells (~3-fold or greater) from lethality; LD_{50} values increased to 25 ± 0.5 μM, 15 ± 0.5 μM, and 8.5 ± 0.4 μM, respectively (Table I). To determine if NAC was directly interacting with β-lap mono(arylimino) derivatives to prevent their conversion to β-lap, we collected UV-vis scans of PIL, MePIL, MPIL, and BPIL, with and without 5 mM NAC, every 30 min for 4 h (Figure 7). We observed that conversion of PIL, MePIL, MPIL, and BPIL to β-lap was blocked (Figure 7*B-E*), while continuous spectral UV-vis scans of β-lap ± 5 mM NAC remained unchanged (Figure 7*A*). NMR analyses confirmed the direct interaction and derivitization of these compounds with NAC (data not shown).

DISCUSSION

Our data support the hypothesis that mono(arylimino) derivatives of β-lap could be potentially useful prodrugs for therapy against human cancers with elevated NQO1 levels. Five derivatives with unique chemical structures (Figure 1, (2)), were directly compared to β-lap and menadione for apoptotic responses and lethality as a function of solubilization in aqueous solutions. Three derivatives (PIL, MePIL, and MPIL) exhibited equivalent, or slightly greater, cytotoxicities to β-lap in $NQO1^+$ compared to $NQO1^-$ cells (Figure 2, Table I). Our results indicated that these β-lap derivatives were Schiff's bases and hydrolyzed to the parent β-lap molecule (Figure 8, (26)). Relative cell growth assays support the theory that these derivatives were converted to β-lap within 4 h after dissolving the compounds in aqueous solutions (Figure 2, Table I), since β-lap-induced cell death was less in NQO1-deficient cells and was inhibited by dicoumarol co-addition in $NQO1^+$ isogenic breast cancer cells (1,18,25). These results were consistent with previous results reported by our group using hollow fiber assays (2), indicating that mono(arylimino) derivatives of β-lap could have an advantage over β-lap.

Direct evidence that specific β-lap mono(arylimino) derivatives spontaneously convert to β-lap in aqueous solution was provided by UV-vis spectrophotometric analyses (Figure 3), NMR analyses (data not shown), HPLC-ESI-MS analyses (Table II), and the preferential lethality responses in $NQO1^+$ compared to $NQO1^-$ cells (Table I). The data presented in Figure 4*A* support our hypothesis that these β-lap mono(arylimino) derivatives need to hydrolyze to β-lap for conversion into a substrate for reduction by NQO1. Furthermore, we noted that the rate of hydrolysis was apparently dependent on the electron-withdrawing strength of the unique substituent

phenylimine group of the compounds (Figures 1, 3, 4, and Table II (26)). The ability of these derivatives to induce NQO1-dependent atypical PARP and p53 cleavage (Figures 5, and 6) confirmed their conversion to β-lap (1,16,18,25). A similar conversion of β-lap mono(arylimino) derivatives to β-lap was observed by Misico et al. (26), where the hydrolytic decay time constants of these same derivatives in aqueous solution were measured by cyclic voltametry. Although never measuring biological activity changes, they concluded that the rate at which these derivatives converted to β-lap increased in acidic solution, and that NPIL and BPIL were more stable than other β-lap mono(arylimino) derivatives at pH 4.5 and 7.4, respectively (26). This enhanced hydrolysis at low pH could be utilized for increased tumor-selectivity.

In contrast, these derivatives were fundamentally different from β-lap since their cytotoxicity was blocked by NAC (5 mM) co-treatments (Table I). Consistent with the ability of NAC to prevent the cytotoxicities of these β-lap derivatives, addition of NAC prevented spontaneous changes (i.e., conversion to β-lap) in the UV-vis spectrophotometric wavelength scans observed with these compounds in aqueous solutions alone (Figure 7). We theorize that NAC directly interacted with these agents to create NAC-derivative conjugates and thereby prevent conversion to β-lap. As a result, NAC exposure rendered the derivatives unable to interact with NQO1 and cause cell death. A similar phenomena of lapachones undergoing sulfur-mediated Michael reactions was previously noted using L-cysteine and β-mercaptoethanol (27).

The current rationale underlying the development of this class of mono(arylimino) prodrugs for the delivery of β-lap for chemo- and/or radiotherapy is based on delayed, pH-dependent activation. After systemic administration of a potent β-lap

mono(arylimino) prodrug, the chemotherapeutic agent must traverse the body through zones of neutral pH via the blood stream and lymphatic circulation, eventually reaching its target, an acidic solid tumor tissue. We believe that these inactive mono(arylimino) derivatives of β-lap would cause minimal damage to normal tissues with neutral pH micro-environments during circulation. Ideally, the prodrug will remain in an inactive form during this initial systemic distribution, and not become active until it successfully reaches the tumor, which commonly has an acidic micro-environment. The converted β-lap is then 'bioactivated' by NQO1 (1), an enzyme found in human tissues at low levels (10,28,29), and expressed at high levels in many human cancers (30-33). We believe that by using some form of a mono(arylimino) prodrug of β-lap, this administered agent would remain inactive in circulation through neutral pH zones until it reached its target tissue (acidic tumors with elevated NQO1 levels), and there it would spontaneously become a high affinity substrate for NQO1 (1). Retention in tumor over normal tissue could be enhanced in NQO1-overexpressing cells due to futile cycling of the converted, and now active, β-lap compound.

The pharmacokinetics of a prodrug are expected to be vastly different from those of the parent drug. This was clearly noted in the development of the camptothecin derivative, CPT-11, which undergoes deesterification *in vivo* to yield SN-38, in the blood of mammals (34). We are in the process of analyzing the pharmacokinetics *in vivo* of β-lap compared to specific β-lap mono(arylimino) derivatives. Depending on the results of the pharmacokinetic studies, we may find that the five β-lap mono(arylimino) derivatives described here are not ideal for therapy against NOQ1-over-expressing human cancers.

PIL, MePIL and MPIL may hydrolyze too quickly at neutral pH, while NPIL and BPIL may hydrolyze too slowly despite being in an acidic tumor environment (26).

Collectively, the data strongly suggest that some form of β-lap mono(arylimino) derivative can be developed for use as a prodrug. The observation that the electron-withdrawing group on the arylimino substitution group can directly influence conversion of the prodrugs to β-lap should be invaluable in developing this active chemotherapeutic agent. Regardless, analyses of this class of β-lap prodrugs could lead to potent, tumor-selective, antitumor agents. We are currently working on developing a β-lap mono(arylimino) compound with a leaving group that is specifically converted to β-lap through a reaction catalyzed by a tumor-selective enzyme. Additionally, we are developing a derivative of β-lap with a cytotoxic leaving group that could further enhance the antitumor efficacy of these prodrugs, particularly in an acidic micro-environment.

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FIGURE LEGENDS

Fig. 1. **Chemical structures.** Shown are structures for menadione; β-lapachone; phenylimine lapachone (PIL); *p*-methylphenylimine lapachone (MePIL); *p*-methoxyphenylimine lapachone (MPIL); *p*-nitrophenylimine lapachone (NPIL); and *p*-bromophenylimine lapachone (BPIL).

Fig. 2. **β-Lap mono(arylimino) derivatives resemble β-lap in mediating NQO1-dependent cytotoxicity.** $NQO1^+$ 231-NQ6 cells were seeded into 48-well plates (4 x 10^4 cells/well) and allowed to attach overnight. Media containing various drug concentrations (β-lap, menadione, PIL, MePIL, MPIL, NPIL, or BPIL) either alone (solid symbols and lines) or co-administered with 40 μM dicoumarol (open symbols and dashed lines) were added for 4 h. Media was then removed, fresh drug-free media added, and cells monitored for changes in relative survival. Relative DNA content per well was determined by Hoechst dye fluorescence, and relative growth (treated/control) plotted as described in "Experimental Procedures." Each point represents the mean ± S.E.M. of three independent wells from three independent experiments.

Fig. 3. **Conversion of β-lap mono(arylimino) derivatives to β-lap.** Stock (50 mM) solutions of β-lap mono(arylimino) derivatives were dissolved in DMSO. Derivatives were then diluted in water (t = 0), and UV-vis wavelength scans (200-500 nm) were recorded every 30 min for 4 h. **(A)** PIL, **(B)** MePIL, **(C)** MPIL, **(D)** NPIL, **(E)** BPIL. Left panels are representative scans of β-lap that remained unchanged over 4 h. Middle panels are scans of β-lap mono(arylimino) derivatives at t = 0 and t = 4 h. Right panels

are overlay scans of β-lap and derivatives at t = 0 and 4 h. Data are representative of experiments performed three times.

Fig. 4. **β-Lap mono(arylimino) derivatives become NQO1 substrates and enter into futile cycling reactions of NADH oxidation similar to β-lap. (A)** NQO1 enzyme activity was assayed as described in "Experimental Procedures" (1) after allowing the β-lap mono(arylimino) derivatives to solubilize in water for 0, 1 or 4 h (t = 0, 1, or 4 h). Each assay was repeated in the presence of 10 μM dicoumarol, and activity attributed to NQO1 was that level inhibited by dicoumarol (5). Reactions were initiated via addition of S9 supernatants derived from NQO1-expressing MCF-7 cells. Enzyme activities were calculated as nmol cytochrome c reduced/min/μg protein. Assays with β-lap and menadione were performed as controls. Results shown are average enzyme activities for three separate cell extractions ± S.D. **(B)** Futile cycling activity was assayed as described in "Experimental Procedures" (1) after dissolving β-lap mono(arylimino) derivatives in water for 0 (t = 0), 1 (t = 1 h) and 4 h (t = 4 h). Reactions were initiated via addition of drug. NADH oxidation was monitored by changes in UV-vis absorbance at 340 nm. Each reaction used an equivalent amount of S9 protein as an NQO1 source as assessed using standard Bradford protein assays. Assays using β-lap and menadione as substrates were performed as controls. Results are expressed as treated/control (T/C) where average moles NADH oxidized/mole substrate (compound)/5 min/μg protein ± S.D. are compared to moles NADH oxidized/mole menadione/5 min/ μg protein ± S.D. Averages were determined from experiments performed three times.

Fig. 5. **β-Lap mono(arylimino) derivatives cause NQO1-dependent, atypical PARP apoptotic cleavage in MCF-7 cells similar to β-lap.** MCF-7 cells were treated with 4 h pulses of **(A)** 5 μM β-lap or 40 μM menadione; **(B-D)** 5 μM PIL, MePIL, or MPIL; or **(E, F)** 20 μM of NPIL or BPIL alone, or in combination with 40 μM dicoumarol (dic) or 5 mM NAC. A higher dose of menadione was necessary to induce apoptosis due to high levels of NQO1 in MCF-7 cells (1). Higher doses of NPIL and BPIL were used to demonstrate their inability to induce apoptosis in $NQO1^+$ cells. Whole cell extracts were prepared at 24 h and analyzed using standard Western analyses as in "Experimental Procedures." Blots were probed with α-PARP antibody and are representative of experiments performed at least three times.

Fig. 6. **β-Lap mono(arylimino) derivatives cause NQO1-dependent atypical p53 apoptotic cleavage in MCF-7 cells similar to β-lap.** MCF-7 cells were treated as in Figure 5 with 4 h pulses of **(A)** 5 μM β-lap, 40 μM menadione or DMSO alone; or **(B)** 5 μM MPIL, alone or in combination with 40 μM dicoumarol (dic) or 5 mM NAC. A higher dose of menadione was needed to induce apoptosis due to the elevated levels of NQO1 in MCF-7 cells (1). Whole cell extracts were prepared 24 h post-treatment and analyzed using standard Western blot analyses as described in "Experimental Procedures." Blots were probed with α-p53 antibody and are representative of experiments performed at least three times.

Fig. 7. **Conversion of β-lap mono(arylimino) derivatives to β-lap is prevented by NAC.** Stock (50 mM) solutions of PIL, MePIL, MPIL, and BPIL were dissolved in DMSO.

Derivatives were then diluted in water (time 0) with or without 5 mM NAC, and UV-vis wavelength scans (200-500 nm) were recorded every 30 min for 4 h. Panel **(A)** is an overlay of scans of β-lap alone at time 0 and 4 h, compared to scans of β-lap mixed with 5.0 mM NAC for 4 h. Panels **(B-E)** are overlay scans of PIL, MePIL, MPIL, and BPIL, respectively, performed as described for panel **(A)** using β-lap. Scans of mono(arylimino) derivatives with 5.0 mM NAC did not significantly change from time 0 to 4 h. Data are representative of experiments performed three or more times.

Fig. 8. **Hypothetical mechanism of β-lap mono(arylimino) derivative conversion to β-lap followed by β-lap redox cycling.** Our data strongly suggest that β-lap mono(arylimino) derivatives undergo a spontaneous Schiff's base hydrolytic reaction, converting the original nontoxic β-lap derivatives into the NQO1-dependent cytotoxic β-lapachone parent compound. The rate of this reaction appears to be dependent upon the electron-withdrawing effect of the R group on the mono(arylimino) phenyl ring (Figure 1). β-Lap undergoes a futile redox cycle with NQO1 wherein the hydroquinone form is unstable, and through two one-electron oxidation steps converts back to the parent β-lap molecule. This cycling "bioactivates" β-lap, and results in a Ca^{2+}-dependent, μ-calpain-mediated apoptotic cell death responses in which p53 and PARP are selectively cleaved (Figures 5, and 6).

Mathematical Equations

$$C(t)=C0+a*(1-\exp(-k*x))$$

$$C(t)=C0+a*\exp(-k*x)$$

Table I.

Comparison of lethality of β-lap analogs in NQO1$^+$ and NQO1$^-$ MDA-MB-231 breast cancer cells by LD_{50} values (μM)

Log-phase cells were treated with a 4 h pulse of compound (± 40 μM dicoumarol or 5 mM NAC). Compound media was replaced with fresh media and the cells allowed to grow for 7 days. Relative DNA per well was determined by Hoechst fluorescence, and relative growth (treated/control DNA) was plotted, as described in "Experimental Procedures."

Compound	Compound Alone		Compound + Dic		Compound + NAC	
	NQO1+[a]	NQO1–[b]	NQO1+	NQO1–	NQO1+	NQO1–
β-Lap	6.6 ± 1.4[c]	14.2 ± 2.1	26 ± 0.5	30 ± 0.5	5.5 ± 0.4	15.3 ± 2.1
PIL	5.9 ± 1.3	16.7 ± 1.8	21 ± 0.5	29 ± 0.5	25 ± 0.5	29 ± 0.5
MePIL	5.7 ± 1.0	15.3 ± 2.3	30 ± 1.0	40 ± 0.5	15 ± 0.5	40 ± 0.5
MPIL	3.7 ± 0.5	9.7 ± 1.3	17.8 ± 1.9	18.0 ± 1.6	8.5 ± 0.4	15.3 ± 1.6
NPIL	>50[d]	>50	>50	>50	>50	>50
BPIL	>50	>50	>50	>50	>50	>50

[a]NQO1$^+$ MDA-MB-231 cells generated as described in "Experimental Procedures."
[b]NQO1$^-$ MDA-MB-231 cells generated as described in "Experimental Procedures."
[c]Table values were determined from relative growth assays performed at least 3 times in triplicate.
[d]Values of >50 μM LD_{50} indicate less than 50% leathality at 50 μM drug, the upper limit of toxic doses used in assaying that drug.

Table II.

Comparison of rate of prodrug hydrolysis to β-lapachone: prodrug loss and β-lapachone formation

Prodrug stock solutions in DMSO were used to prepare methanolic working solutions that were diluted 10:1000 v/v in water (pH ~5.8, 30 °C). Aliquots were injected into the HPLC-ESI-MS system every hour for 30 h. Loss of prodrugs and formation of β-lap were monitored as described in "Experimental Procedures."

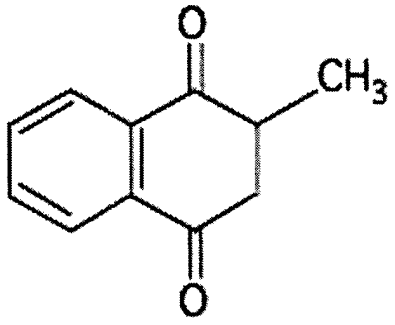
	Prodrug loss		β-Lapachone formation		
Compound	k^a	$k_{rel}{}^b$	k^a	$k_{rel}{}^b$	$t_{1/2}{}^c$
PIL	–0.154	1.0	0.097	1.0	7.15
MePIL	–0.549	3.6	0.208	2.1	3.33
MPIL	–0.693	4.5	0.221	2.3	3.14
NPIL	–0.001	0.0	0.034	0.4	20.39

[a]First order rate constant hr^{-1}

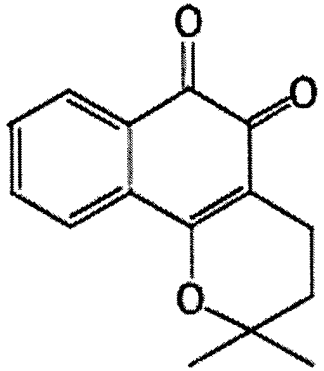
[b]Rate relative to PIL's rate, defined as k(drug)/k(PIL)

[c]Half time of first order process with rate constant k calculated as $t_{1/2} = \ln(2)/k$

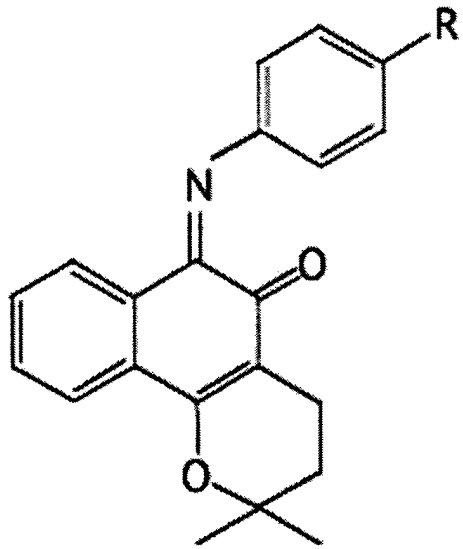
Figure 1



menadione



β-lapachone



R = H, phenylimine (PIL)
R = CH_3, *p*-methylphenylimine (MePIL)
R = OCH_3, *p*-methoxyphenylimine (MPIL)
R = NO_2, *p*-nitrophenylimine (NPIL)
R = Br, *p*-bromophenylimine (BPIL)

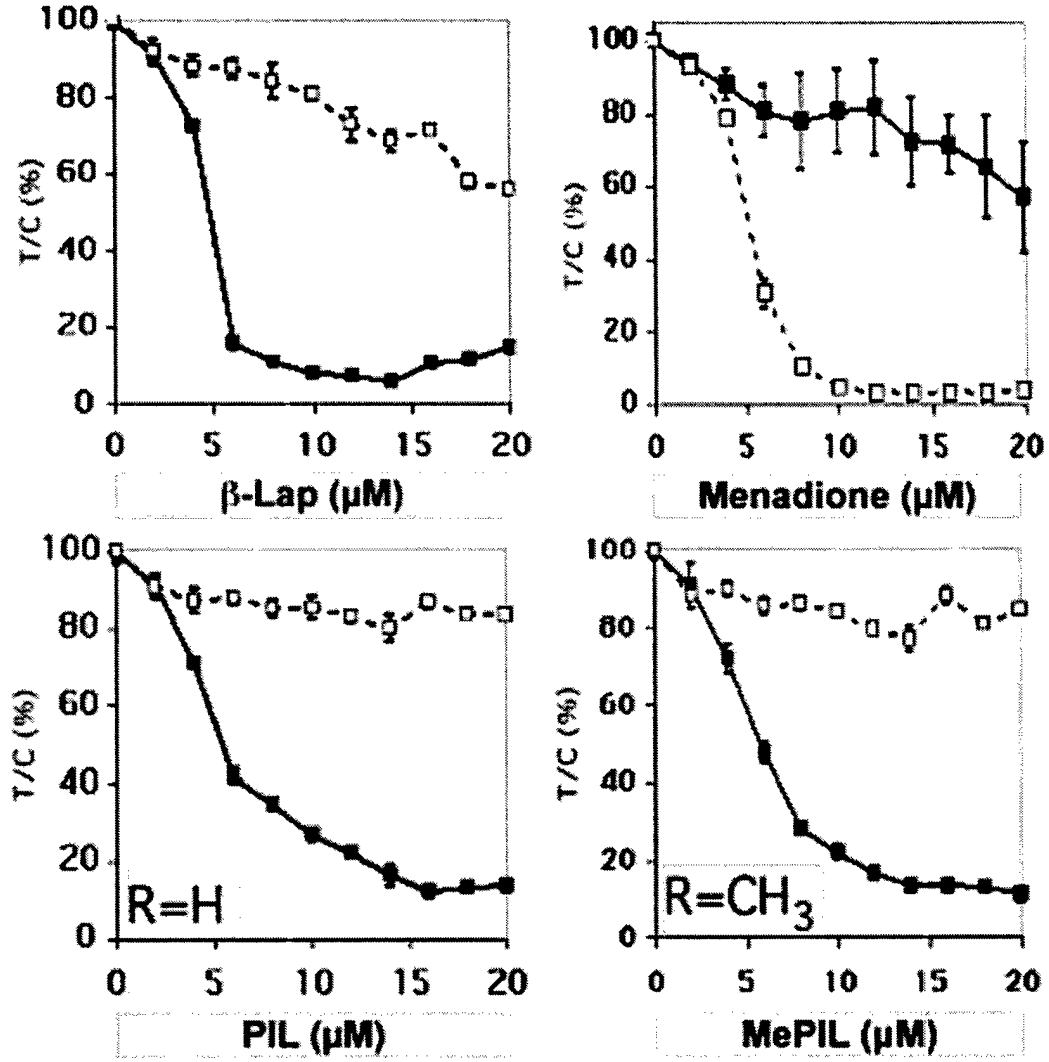


Figure 2

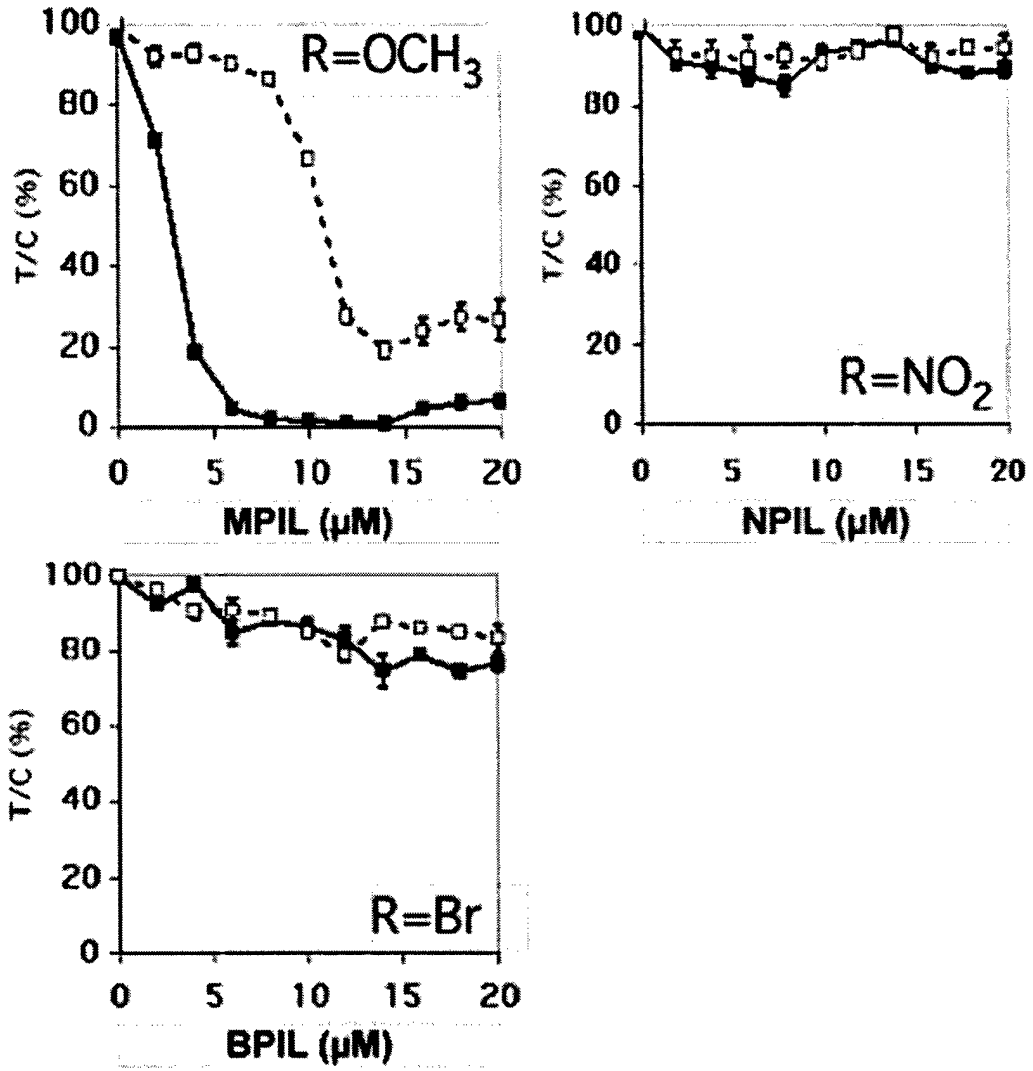

R=OCH3
T/C (%)
MPIL (μM)
R=NO2
NPIL (μM)
R=Br
BPIL (μM)
0
20
40
60
80
100
0
5
10
15
20

Figure 2

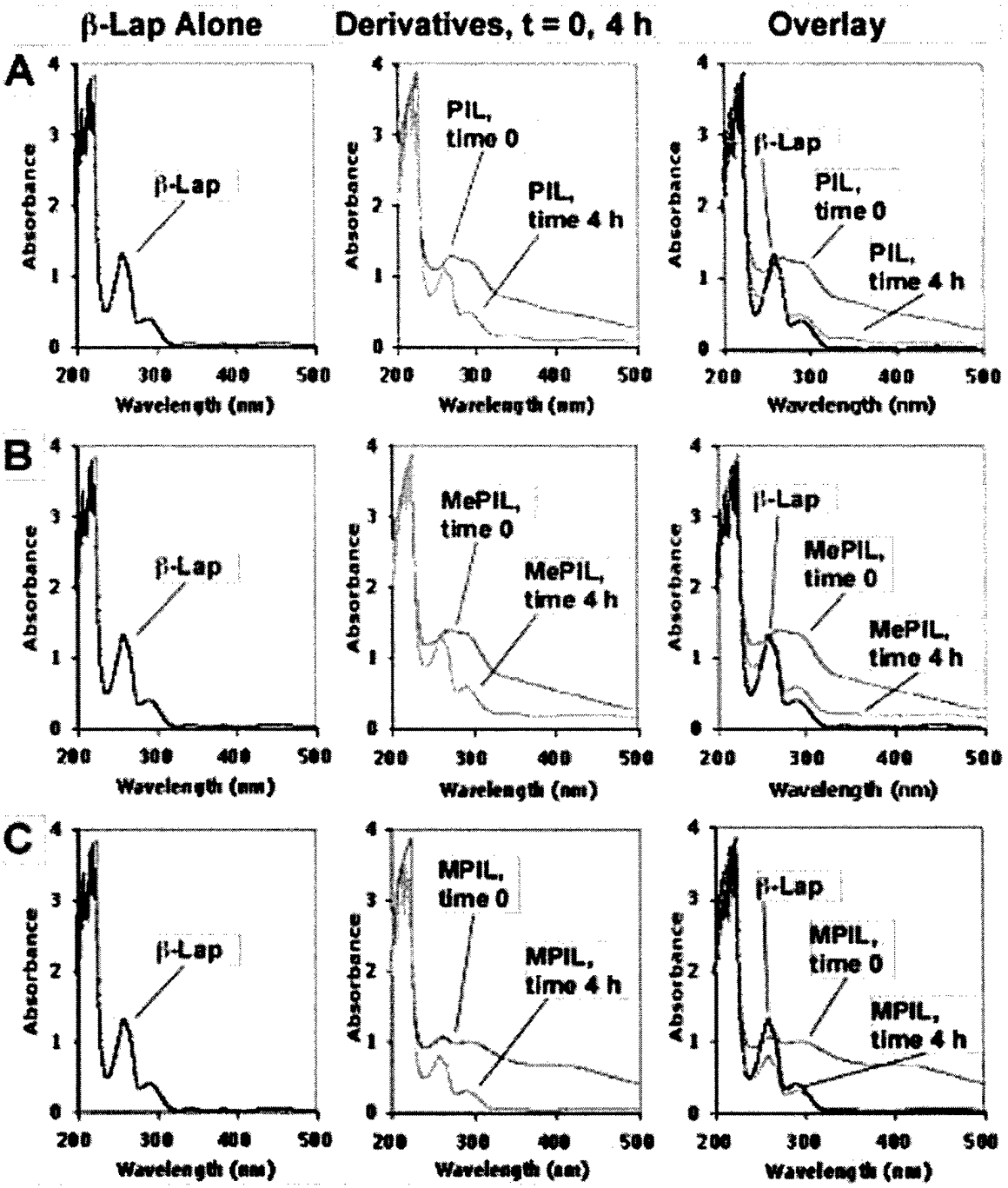

β-Lap Alone
Derivatives, t = 0, 4 h
Overlay
A
B
C
Absorbance
Wavelength (nm)
β-Lap
PIL, time 0
PIL, time 4 h
MePIL, time 0
MePIL, time 4 h
MPIL, time 0
MPIL, time 4 h

Figure 3

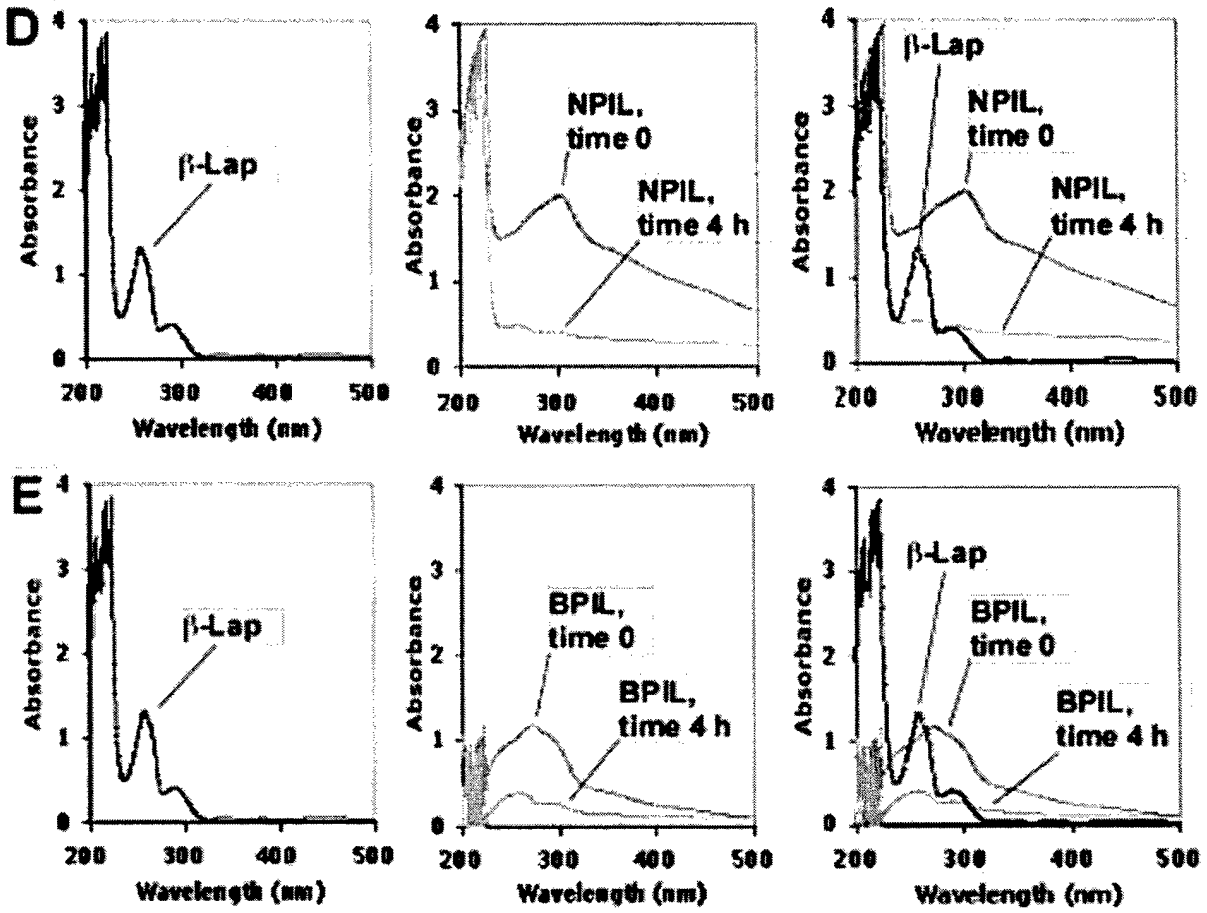

D
Absorbance
Wavelength (nm)
β-Lap
NPIL, time 0
NPIL, time 4 h
E
BPIL, time 0
BPIL, time 4 h

Figure 3

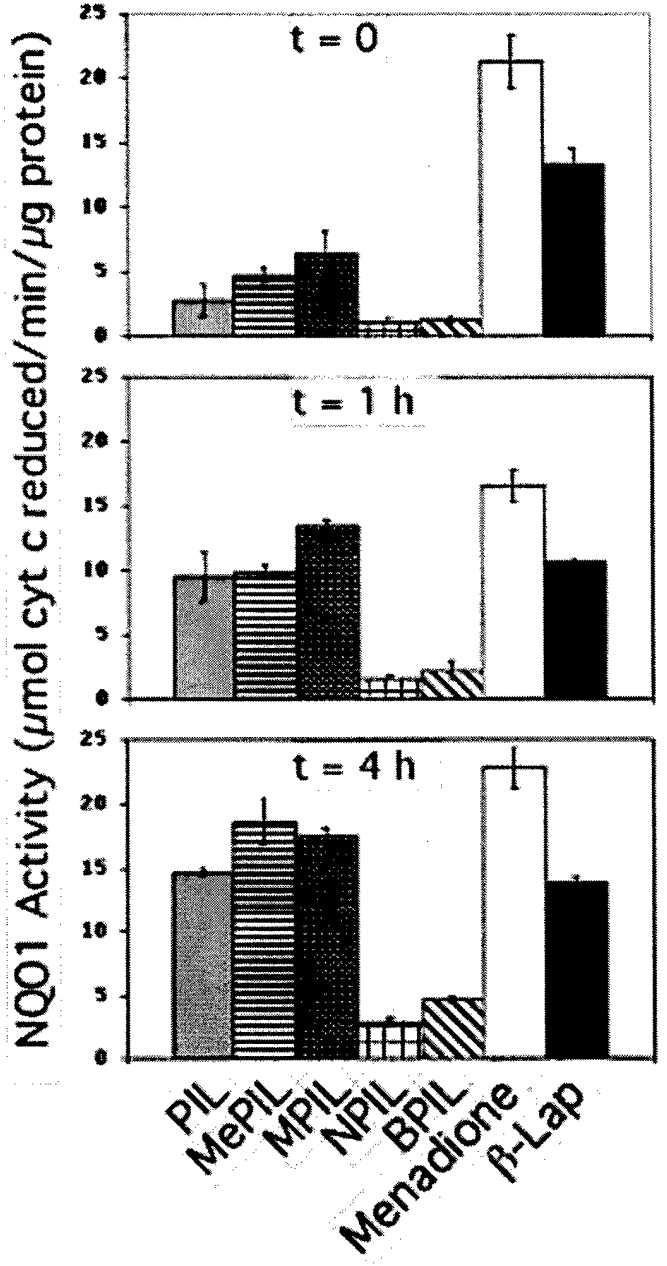

t = 0
t = 1 h
t = 4 h
NQO1 Activity (μmol cyt c reduced/min/μg protein)
0
5
10
15
20
25
PIL
MePIL
MPIL
NPIL
BPIL
Menadione
β-Lap

Figure 4A

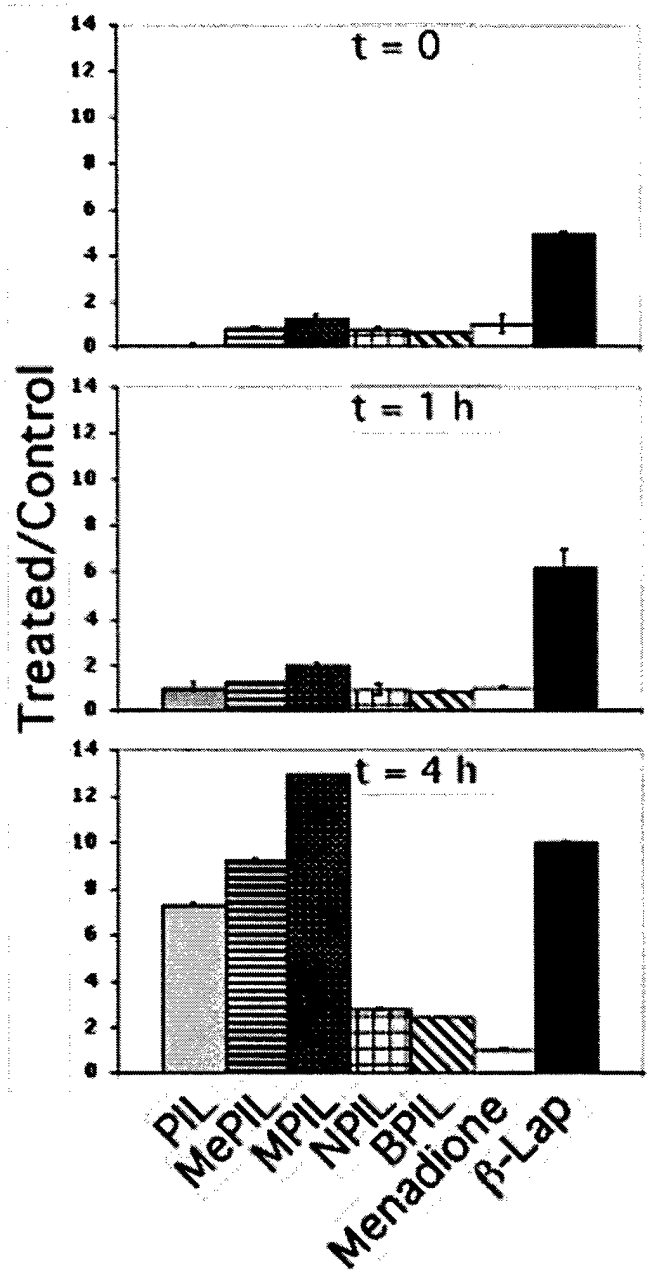
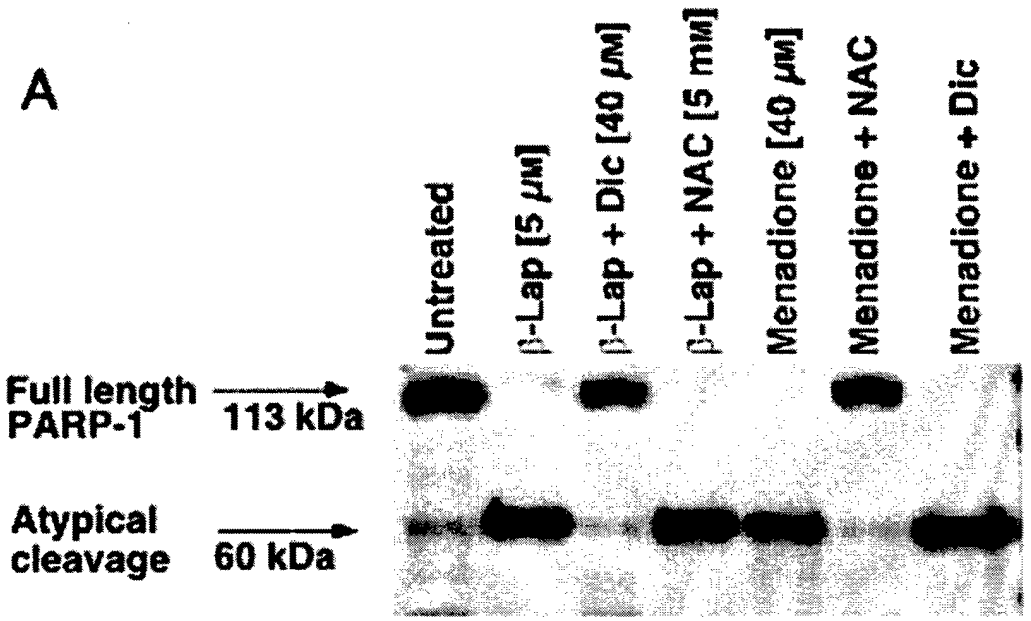

t = 0
t = 1 h
t = 4 h
Treated/Control
0
2
4
6
8
10
12
14
PIL
MePIL
MPIL
NPIL
BPIL
Menadione
β-Lap

Figure 4B


A
Untreated
β-Lap [5 μM]
β-Lap + Dic [40 μM]
β-Lap + NAC [5 mM]
Menadione [40 μM]
Menadione + NAC
Menadione + Dic
Full length PARP-1
113 kDa
Atypical cleavage
60 kDa

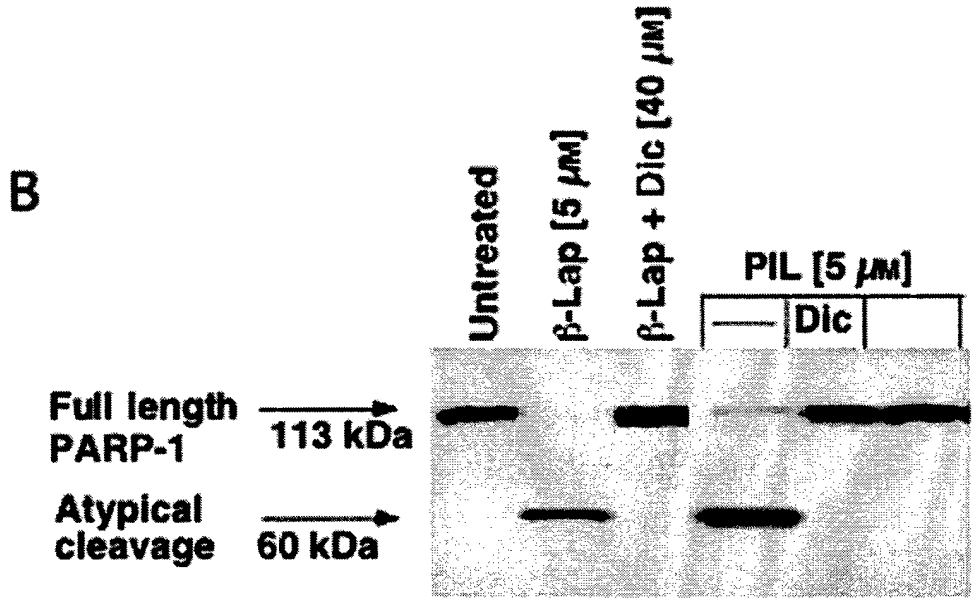

B
Untreated
β-Lap [5 μM]
β-Lap + Dic [40 μM]
PIL [5 μM]
—
Dic
Full length PARP-1
113 kDa
Atypical cleavage
60 kDa

Figure 5

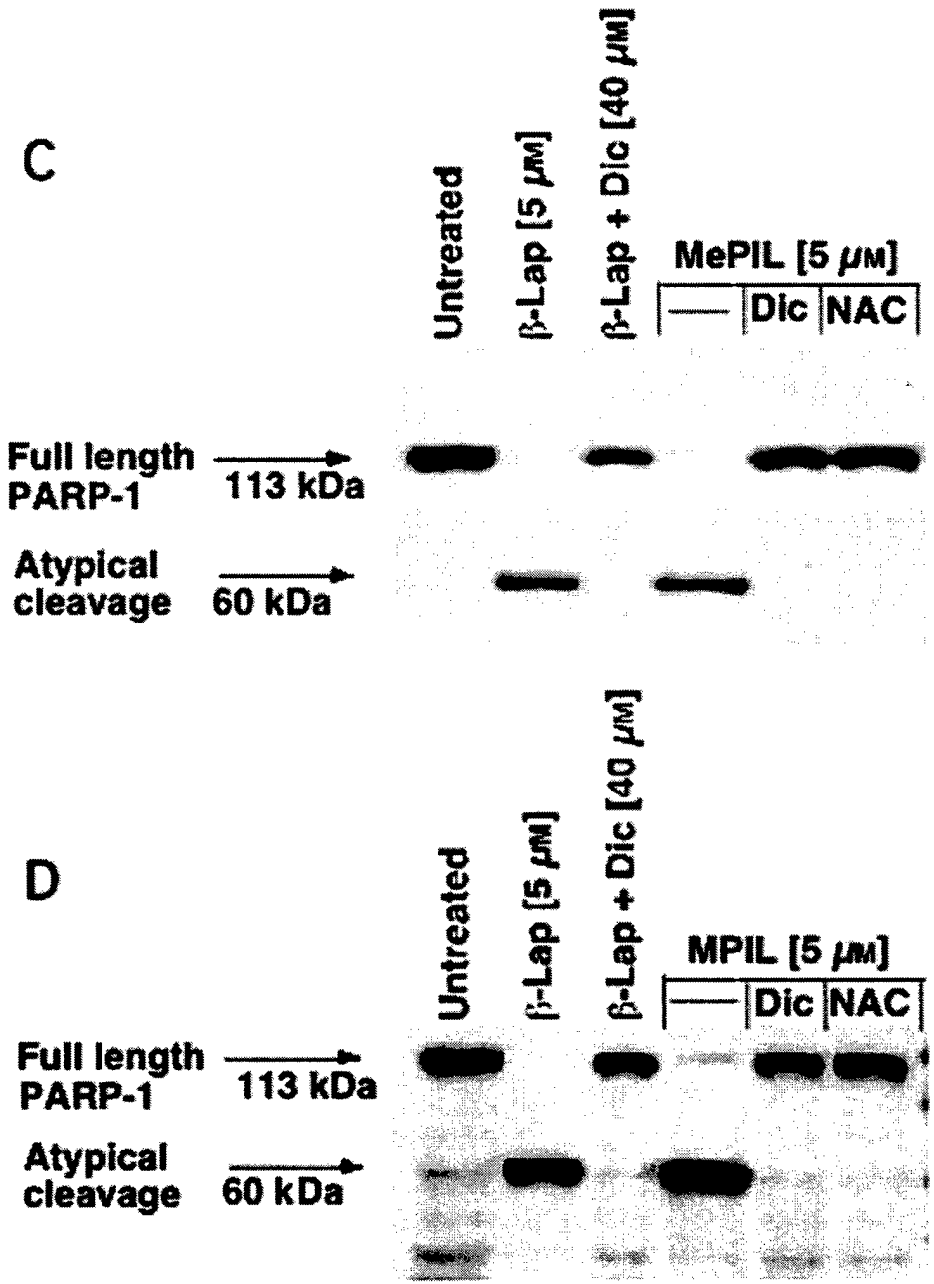

C
Untreated
β-Lap [5 μM]
β-Lap + Dic [40 μM]
MePIL [5 μM]
—
Dic
NAC
Full length PARP-1
113 kDa
Atypical cleavage
60 kDa
D
Untreated
β-Lap [5 μM]
β-Lap + Dic [40 μM]
MPIL [5 μM]
—
Dic
NAC
Full length PARP-1
113 kDa
Atypical cleavage
60 kDa

Figure 5

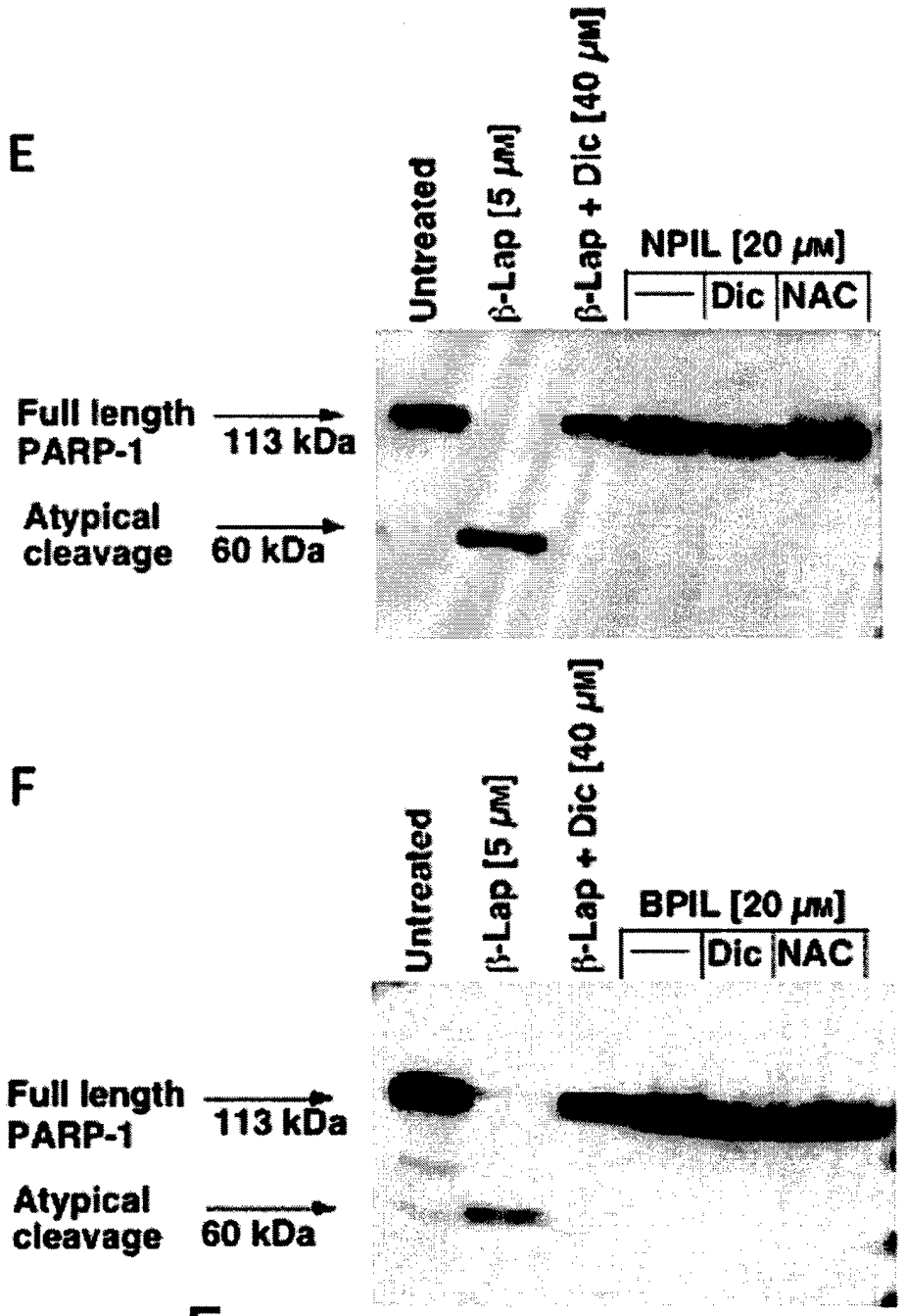

E
Untreated
β-Lap [5 μM]
β-Lap + Dic [40 μM]
NPIL [20 μM]
—
Dic
NAC
Full length PARP-1
113 kDa
Atypical cleavage
60 kDa
F
Untreated
β-Lap [5 μM]
β-Lap + Dic [40 μM]
BPIL [20 μM]
—
Dic
NAC
Full length PARP-1
113 kDa
Atypical cleavage
60 kDa

Figure 5

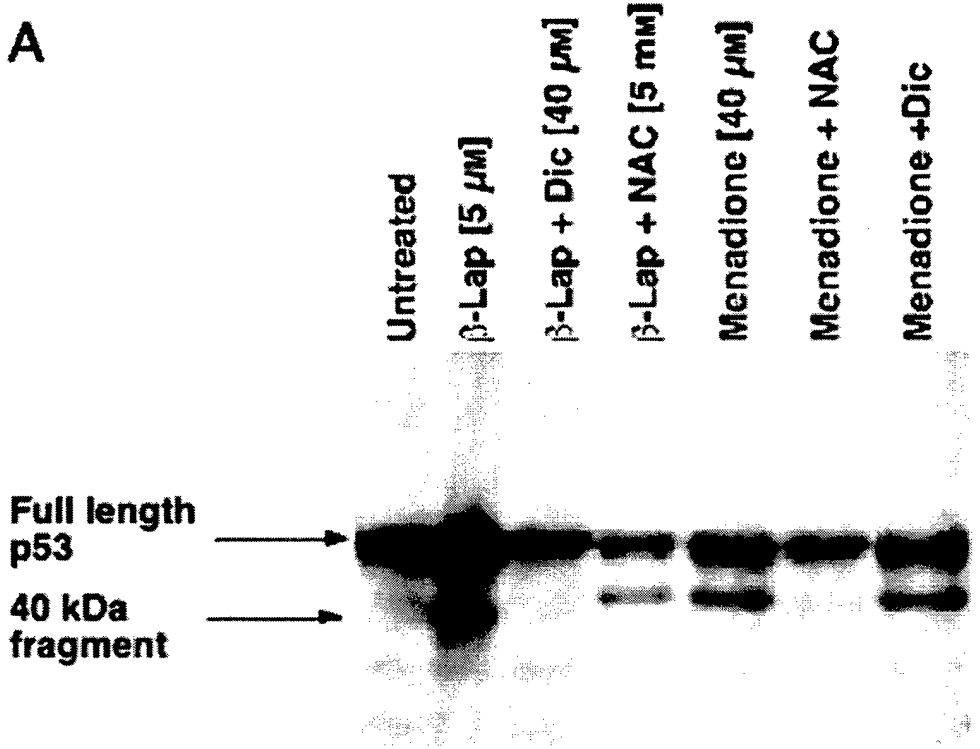

A
Untreated
β-Lap [5 μM]
β-Lap + Dic [40 μM]
β-Lap + NAC [5 mM]
Menadione [40 μM]
Menadione + NAC
Menadione +Dic
Full length p53
40 kDa fragment
B
Untreated
β-Lap [5 μM]
β-Lap + Dic [40 μM]
MPIL [5 μM]
—
Dic
NAC
Full length p53
40 kDa fragment

Figure 6

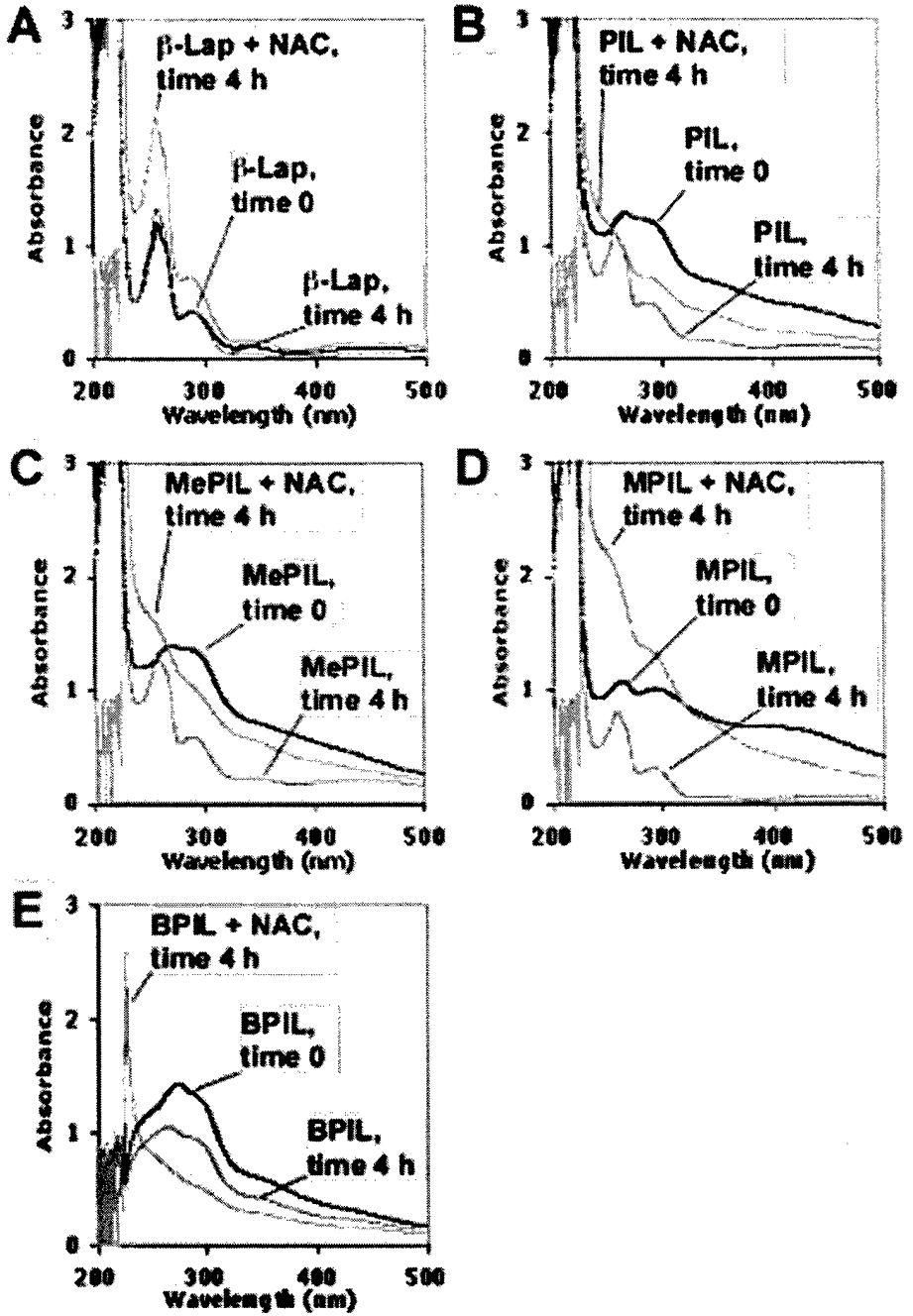
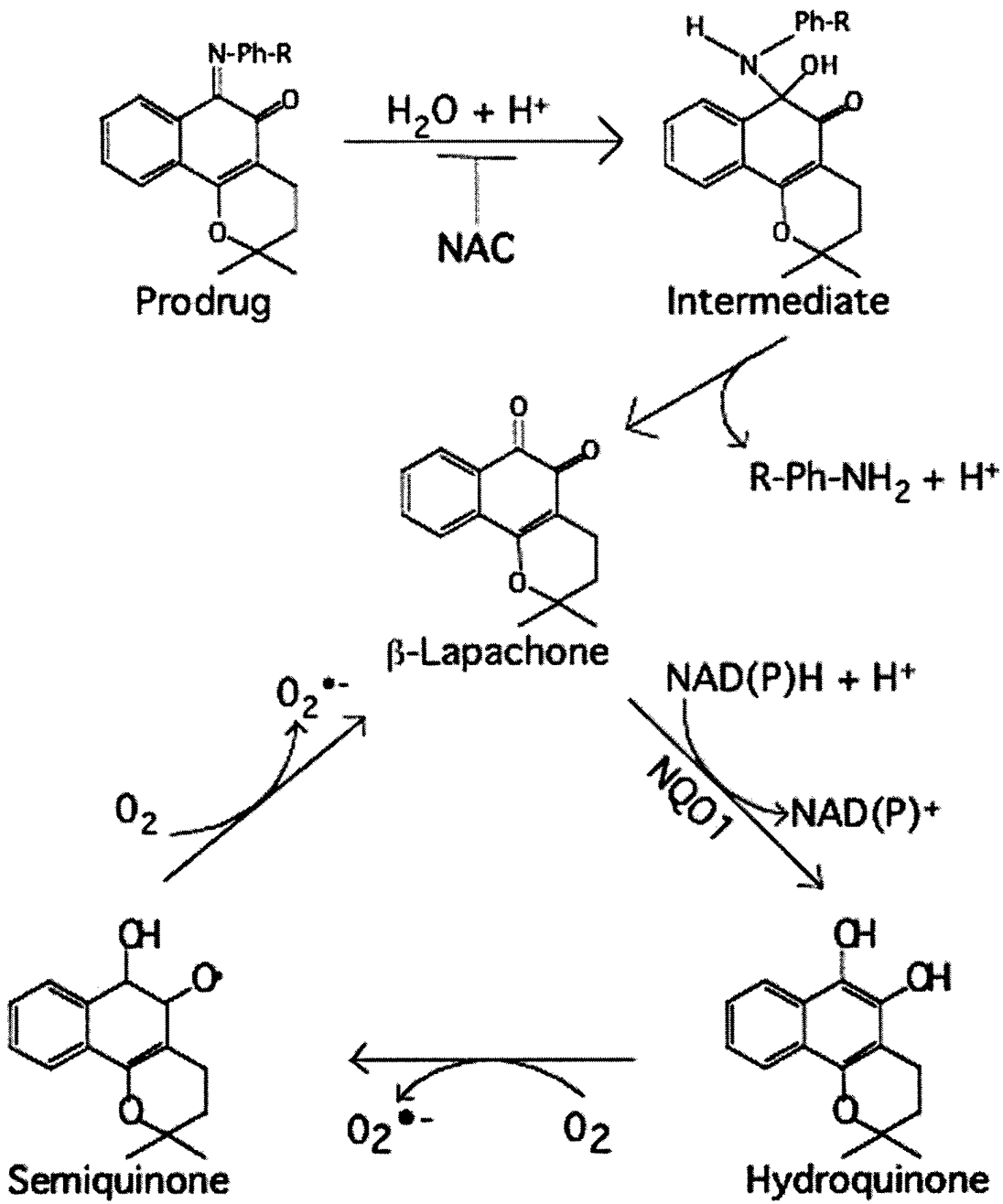

A
β-Lap + NAC, time 4 h
β-Lap, time 0
β-Lap, time 4 h
B
PIL + NAC, time 4 h
PIL, time 0
PIL, time 4 h
C
MePIL + NAC, time 4 h
MePIL, time 0
MePIL, time 4 h
D
MPIL + NAC, time 4 h
MPIL, time 0
MPIL, time 4 h
E
BPIL + NAC, time 4 h
BPIL, time 0
BPIL, time 4 h
Absorbance
Wavelength (nm)
0
1
2
3
200
300
400
500

Figure 7

Figure 8


N-Ph-R
O
O
Prodrug
$H_2O + H^+$
NAC
H
Ph-R
N
OH
O
O
Intermediate
R-Ph-NH_2 + H^+
O
O
O
β-Lapachone
NAD(P)H + H^+
NQO1
NAD(P)$^+$
$O_2^{\bullet-}$
O_2
OH
O
O
Semiquinone
$O_2^{\bullet-}$
O_2
OH
OH
O
Hydroquinone